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Toxicological and analytical assessment of e-cigarette refill components on airway epithelia

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ABSTRACT

There are over 2.6 million users of e-cigarettes in the United Kingdom alone as they have been promoted as a safer alternative to traditional cigarettes. The addition of flavours and aromas has also proven to be popular with younger generations. In this review, we survey the range of studies in the short timeframe since e-cigarettes reached the market to draw attention to the health associated risks and benefits of their introduction. We complement this review with a case study reporting on the composition of selected e-cigarette refills with particular emphasis on the toxicological activity of its components on lung cells.

Keywords: *e-cigarettes, flavours, Beas2B, toxic*

1. Background

Electronic cigarettes (e-cigarettes, e-cig or personal vapouriser [PV]) are battery-powered devices that deliver vapourised chemicals to the user. They have achieved sales of over $1.7 billion for 2013[1], and currently there are over 7,500 flavour variations available[2]. There are over 2.6 million users of e-cigarettes in the United Kingdom[3]. They may contain nicotine alongside other chemicals, such as flavourings and enhancers, while some variants may contain tobacco extracts[4]. The key differences between conventional and e-cigarettes are that e-cigarettes do not usually contain tobacco[5], and smoking conventional cigarettes leads to the combustion of tobacco products. The process of heating in e-cigarettes is gentler than in conventional cigarettes[5]. Several studies clearly show that e-cigarettes' vapours have less combustion products than the ones produced by regular cigarettes, many of which are carcinogenic[5], though new manufacturers are increasing the heating temperature to allow for a more 'real' effect[6]. As 30% of the cancer deaths in USA are caused by tobacco, and from this more precisely the tar component is the killer[7,8], it is understandable that e-cigarettes (with no tar available) are being branded as a safer alternative to tobacco.

E-cigarettes are composed of a cartridge or tank which is used to store liquid material containing the 'e-liquid', 'e-juice' or 'nicotine solution'[9]. The cartridge serves as a reservoir of storage for the liquid and also acts as the mouthpiece of the e-cigarette. A heating element is used as an atomiser to turn the liquid into a vapour[10], and a power source such as a battery, which can be either manual or automatic, make up the rest of the device. The vapour is only produced while the heating element is activated and not between puffs. The vapourised liquid condenses into an aerosol, later inhaled delivering nicotine, vehicle and flavourings[9,11]. The vapour is generated by heating the solution to temperatures ranging from 65 °C to 120 °C, with a reported maximum atomiser temperature of approximately 250 °C[9] increasing the chances of carbonyl formation. Different models are available with some more manual types to control the delivery and temperature[12]. Propylene glycol and glycerine are used as carriers with the first one being the more widely employed, even though glycerine has been used in traditional cigarettes[13]. The vapour can contain carbonyl compounds like formaldehyde, acetaldehyde, and acrolein, which have been shown in numerous

studies to be toxic. Formaldehyde and acetaldehyde are classified as carcinogens[14,15] and acrolein as an irritant[16].

E-cigarettes are sold as a healthier option to tobacco smoke and physicians are currently being asked for their opinions in this area[17]. Furthermore, around 95% of the general population believe them to be healthier[18]. So far research has proven that the e-cigarette vapour is not benign, but less hazardous than traditional cigarettes[17]. E-cigarette users commented in open internet forums on side effects for users such as headaches, respiratory tract irritations and digestive problems[17]. Clinical studies has shown only 10% of traditional smokers quit smoking after switching to e-cigarettes, but the biggest change was observed in the reduction of traditional cigarettes per day in favour of e-cigarette puffing[18]. On the pro e-cigarette side, the hypothesis postulates the absence of the tar products, pyrolysis and lower plasma nicotine content (around 10% of the tobacco cigarette) would make it a healthier option for traditional cigarette smokers. A clinical study has also shown that cell blood counts and markers are statistically unaffected by exposure of e-cigarette users and passive users. On the contrary, exposure to tobacco cigarettes (users and passive users) results in markers of inflammation after 3 h[19].

However, there is conflicting information regarding the risks posed to public health and the health benefits from e-cigarettes. The Consumer Advocates for Smoke-free Alternatives Association (CASAA) has reported a significant risk reduction when assessed against regular cigarettes[20]. However, a number of studies indicate that e-cigarettes may produce long-term and short-term side effects, such as airway resistance, irritation of the airways, redness of the eyes and drying out the throat[21–23]. Research has been focused on the toxicological aspect of e-cigarettes on the lungs, the heart and cancer[1] while some reports might have inconsistencies or conflict of interest, though the general view directs towards a more toxic effect[24]. The World Health Organisation (WHO) and the Food and Drug Administration (FDA), have indicated the safety and the potential health damage of e-cigarettes and its constituents have not been fully studied and so remain undetermined[10,25]. Guidelines from the FDA indicate an inclination towards the enforcement of the same rules applying to traditional cigarettes for the term of sales and marketing strategies of electronic cigarettes[1].

The majority of the research has been divided as follows: (i) analytical assessment of the e-liquids; (ii) analytical assessment of the vapour phase; (iii) toxicity of the e-liquids and/or vapour in animal models and/or animal cells; (iv) toxicity of the e-liquids and/or vapour in human cells (primary and immortalised both cancer and non-cancerous, 2-dimensional and 3-dimensional); and (v) clinical studies on cigarette (traditional and/or e-cigarettes) smokers. Though the analytical assessment seems to be more reproducible due to standardising methods used in the chromatographic method, eluents and detection, more variability appears in the biological work. This might be related to the dosing, concentration of ingredients, sample variation from same or different manufacturers, flavourings, cells and even different cell culture media used to feed cells.

2. Composition

Tobacco smoke comprises many classes of chemicals including polycyclic aromatic, alkaloids (such as nicotine), hydrocarbons and benzo(a)pyrenes[26]. The green leaves of the tobacco plant are almost entirely free of the dangerous tobacco specific nitrosamines (TSNA) such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and N'-nitrosonornicotine (NNN); during the post-harvest process (known as curing) these TSNA are formed when the natural occurring alkaloids are nitrosated. These chemicals can then enter the respiratory system during cigarette burning[27,28]. There is a wide difference in the content of TSNA in different tobacco brands, implying different plant sources, curing and purifications process[29]. E-cigarettes do not have a source of combustion, this is one of the reasons why the health risks of vaping are assumed to be less harmful compared with traditional smoking. Therefore manufacturers have shown a growing interest to produce e-cigarettes for indoor use, where traditional cigarettes have been banned[20,30]. Nevertheless some studies indicate, in general, that the components in e-cigarette aerosols and e-liquid refills contain: the carbonyls formaldehyde (up to 9.0 μg g^{-1} of e-liquid); acetaldehyde (up to 10.2 μg g^{-1} of e-liquid); acrolein (up to 5.5 μg $puff^{-1}$); propionaldehyde (up to 1500 ng $puff^{-1}$); the volatile organic compounds (VOCs) toluene (up to 6.3 μg 150 $puff^{-1}$); N-nitrosonomicotine (NNN) (up to 16.7 ng mL^{-1} e-liquid); 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (up to 10.8 ng mL^{-1} e-liquid); glycols such as propyelene glycol and glycerine (variation); nicotine (depending on the manufacturer's label); traces of polycyclic aromatic hydrocarbons and the metals Ni (up to 0.29 μg 150 $puff^{-1}$); Cd (up to 0.22 μg 150 $puff^{-1}$); and Pb (up to 0.57 μg 150^{-1} puff) with traces of Ag, Al, Zn and Cr[18,31].

2.1. Nicotine

From vapours containing tobacco, tobacco specific nitrosamines (TSNAs) including NNN and NNK can be formed in the combustion process in traditional cigarettes[32] and are considered to be highly toxic[33,34]. There is evidence these toxic carbonyl compounds have been found in the vapour of e-cigarettes[35]. Some studies have demonstrated that impurities and nicotine degradation products such as nicotine-*cis*-N-oxide, nicotine-*trans*-N-oxide, myosmine, anabasine, and anatabine, which are very carcinogenic, can be found in e-cigarette refill liquids[36]. The molecules can lead to mutations in genes such as Ras (vital function in signal transduction of cell proliferation), p53 and Retinoblastoma (with roles as tumour suppressors) as these molecules can form adducts with cellular DNA[37–40]. Nicotine can be absorbed through different routes such as inhalation, ingestion, skin, and mucous membranes, therefore it is feasible that the vapour from e-cigarette users could cause secondary exposure of nicotine and other toxins to the individuals in the surrounding area[26]. Nicotine is a stimulant and side effects can include death. A danger of e-cigarette refills are that children can mistake the

bottles containing fruity or sweet flavours and aromas for fruit juices; a fatality on a 2 year old child after drinking an unknown amount of e-cigarette refill has been reported[12]. Concentrations of nicotine in the air have been studied for conventional and e-cigarettes. It has been reported that e-cigarettes with a refill liquid of nicotine concentration of 24 mg mL^{-1} emitted nicotine concentrations between 0.82 μg m^{-3} to 6.23 μg m^{-3}, with the mean concentration of nicotine from regular cigarettes 10 times higher (31.60±6.91 μg m^{-3})[41,42]. A threshold limit of nicotine exposure in the work place has been published by The American Conference of Governmental Industrial Hygienists which established a limit of 500 μg m^{-3} for an 8 h time-weighted average (TWA)[43]. Manufacturers indicate the expected level of nicotine in the e-cigarette refills, and though they are very close to the label value, some samples seem to not reflect this value[32]. We observed in our studies that the provenance of nicotine is also an important factor. Nicotine can be used in the chemical industry and this grade of nicotine is not as pure as pharmaceutical nicotine which should be employed in the tobacco industry.

Alkaloids are present in plants and one of the most notorious examples is nicotine. Other alkaloids from tobacco can include cotinine, myosmine and anabasine, all of which are present in e-cigarette refills. A comprehensive study on the alteration of gene expression on CCL-185 (human lung carcinoma cell line) upon exposure to these four alkaloids[44] showed up-regulation of CEACAM6 (adhesion molecule involved in carcinogenesis and metastasis) when the cells were treated with nicotine and myosmine and decreased when exposed to anabasine and cotinine. In the case of ALDH3A1 (an enzyme involved in the detoxification of reactive aldehydes), the treatment with myosmine showed up-regulation while PIR (transcription regulator for apoptosis and oxidative stress) was down-regulated in the cases of nicotine, anabasine and cotinine, and mysomine had little effect. Only nicotine showed up-regulation of TLR4 (ligand involved in the immune response) while the other compounds showed a decrease thereof[44].

2.2. Various metals

Metals such as nickel, cadmium, lead and silica particles can be present in the aerosols produced from e-cigarettes. They could arise from the wick and heating coil constituents, and are considered to be carcinogenic, nephrotoxic, neurotoxic, and hemotoxic[45].

2.3. Glycerine (glycerol) and propylene glycol

Glycerine is an intermediate in carbohydrate and lipid metabolism. It is used as a solvent, emollient, pharmaceutical, and sweetening agent in the food industry[46]. Both glycol and glycerine are used in manufacturing industries as well as aviation and are well known respiratory irritants[32]. Glycerine and propylene glycol are chemical compounds both used in normal and e-cigarette liquids to control the moisture content[47]. However, they may be pyrolysed (burned) to acrolein and formaldehyde

at higher temperatures[15]. Acrolein and formaldehyde have been found in e-cigarette vapour even though the levels detected were 15 times smaller than conventional cigarettes. This is due to the fact that the evaporation temperature of e-liquids at 100–250 °C[48] is lower than that of the combustion temperature of up to 650 °C in regular tobacco cigarettes[49,50].

2.4. Flavourings and their toxicities

The sensation of flavours is determined by chemical substances that can interact with the senses of taste and smell[51]. There are over 2,500 individual flavouring substances employed in the food industry. Safety procedures have been introduced to control their use[52], though they are directed to a consumption through food rather than e-cigarettes where the uptake is different. In general, through oral consumption, quantities in food need to be considered and these chemicals (aldehydes, esters and acids) tend to be metabolised very rapidly through active enzymes in the liver and intestine (phase I and phase II enzymes, including the CYP450 family and glutathione transferase)[52]. A decision tree is followed based on the chemical structure and concerns on data from human and animal studies. The Flavour and Extract Manufacturers Association (FEMA) assess the safety of chemical compounds used as flavouring ingredients but cannot regulate the use of the flavour ingredients in e-cigarettes, as the use of the flavourings in e-cigarettes has not been approved[53]. Some anti-tobacco groups claimed the addition of flavours to traditional cigarettes could attract new smokers; different flavours can be added from oils to natural extracts with the majority of them in the fruity range such as mint and menthol. In the case of traditional cigarettes, the combustion temperature could produce pyrolysis or oxidation of these compounds converting them into toxic carbonyls[54]. There is often no more information given about the composition or source of such additives, other than that, these flavours are 'natural'[20]. As the most widely available sources of flavourings are for food products, we could expect some of the e-cigarette manufacturers could be using food flavouring products. For example, diacetyl (butanedione or butane-2,3-dione) is a by-product of the transformation of glucose to ethanol by yeast during the beer fermentation process and is extensively used in the food industry giving flavour to dairy products[55]. It is safe as a food flavouring in popcorn, but when inhaled it has been shown to produce 'popcorn lung syndrome' or *bronchiolitis obiliterans*[56]. Animal studies of diacetyl exposure have shown morphological changes in the liver[55] and studies of cells exposed to butterscotch flavoured e-cigarettes have also shown toxicity[21].

Menthol is one of the most widely used flavours in both e-cigarettes and traditional cigarettes. These mentholated (e)-cigarettes seem to mask some early signs of respiratory diseases as menthol has antitussive properties. Nevertheless, this seems to be more a hypothesis than real data[57] and very limited information of toxicological data is available even on traditional cigarettes[58]. Menthol is a volatile compound and it could be readily vapourised rather than suffer pyrolysis. As a

food additive, menthol has been subjected to much toxicological research but little has been done on the respiratory tract with very little findings besides irritation *in vivo*, even though menthol is present in many products directed to treat respiratory problems such as the case of Vicks Vaporub.

An interesting study[59] on an adenocarcinomic human alveolar basal epithelial cell (A549, a cancer cell line, generally used for anticancer drug screening safety profiling of new drugs) showed that exposure of vapours of several flavouring agents, cinnamladehyde, benzaldehyde, diacetyl, 2,3-pentadione, vanillin, acetoin and triacetin, for 24 h proved to be very toxic on the cells; especially in the cases of cinnamaldehyde and benzaldehyde. This is in accordance with other studies where cinnamon flavour in e-liquids has shown high cytotoxicity levels in other cells[34]. Interestingly, vanillin, acetoin and triacetin proved to be the least toxic. Another study employing the same cell line but challenged to different varieties of e-liquids found no toxicity though there was an increased level on the release of IL-8 but only at a very high dose[60].

Immortalisation of human cells using telomerase or SV40 virus are a good option for cytotoxic studies. In the case of bronchial cells, some examples are Beas-2B and 16-HBE14o. A study testing individual flavours for chocolate (2,5-dimethypyrazine), vanillin, apple/citrus (damascenone), floral (linalool), raspberry (α-ionone), sweetener (ethyl maltol, generally used for candy floss, caramelised sugar) and strawberry furaneol challenged the cells for 24 h. At the concentrations tested, findings showed vanillin and furaneol were relatively non-toxic (in agreement with other studies)[59]. The rest of the flavours showed activity on the cells while the chocolate flavour showed a reduction of the capability of producing/communicating signalling molecules[2].

As the e-cigarette industry is growing, more needs to be done to assess the quality of the ingredients as well as the biological effects. Many groups compare both the analytical composition and the toxicological effect of the e-liquid and the vapours associated with it[61,62]. The different temperatures that can be achieved in the vapourisation chamber (up to 350 °C) can modify the functionality of the chemical ingredients transforming them into dangerous carbonyls such as formaldehyde and acetaldehyde. Their concentration in the vapour seems to be dependent on the voltage and temperature[62], nevertheless the amount of nicotine found in the aerosol has been found to be 85% lower than traditional cigarettes[63] implying the smoker and passive smokers of e-cigarettes are exposed to less damage.

A very interesting decision tree for carcinogenicity, mutagenicity and teratogenicity for flavourings proposed by Costigan[61] highlights the need to compare both what the seller informs about the ingredients and their quantities with the results from the e-liquid and breakdown products employing GCMS. From here the ingredients can be compared to an existing database for biological information. If more and/or new ingredients are found then they will need to be assessed.

3. Analytic method of assessment

Gas-chromatography coupled to mass spectrometry, GCMS, is the most popular method for analytical detection in the majority of the articles revised. Other variants include gas chromatography coupled to thermal energy analysis (GCTEA) which is very sensitive to nitrosamines[63] and liquid chromatography with tandem mass spectrometry (LCMSMS). Each technique has benefits and disadvantages. The ingredients in the e-liquids are volatile compounds and some e-liquids have a simple formulation (single flavouring agents, propylene glycol and nicotine) and some have complex mixtures (natural extracts for flavour, sweeteners, tobacco and more). GC relies on the volatility of each chemical and when this is not possible, a process of derivatisation can be added. LC on the other hand, does not require the compounds to be volatile but to dissolve in the elution system, generally using acidified water/methanol or water/acetonitrile with the pH modified to aid separation and ionisation. Columns used in both methods are generally based on C18 for LC[64], polar column such as HILIC have been very useful and polysilane for GC[65]. We found some compounds such as menthol are not detected very well in LCMS though it is easily observed using GCMS. The tandem quadruple MSMS or time of flight (ToF) allows for quantification if a patron was used for compound monitoring (optimisation technique that works as a fingerprint). Limits of detection and sensitivity apply to all techniques as well as accuracy[63]. Some groups have used both GCMS and LCMS but for different purposes[66], for example, GCMS for the analysis of solvents/humectants (propylene glycol and glycerine) and polycyclic aromatic hydrocarbons, and LCMS for the quantification of nicotine, nitrosamines and flavours.

From the review data, there is no 'one fits all' but more modern equipment seems to perform better than some of the older techniques. Infra-red (IR) technology can detect the functional groups in small molecules and can differentiate if in a sample there is a carbonyl, a hydroxyl or a nitrile group. New and more sensitive equipment using IR is emerging and allowing for the use on the cosmetic, food, and forensic industries[67–70]. Techniques like Attenuated Total Reflectance – Fourier Transform IR (ATR-FTIR) and near IR (NIR) alongside modelling methods like K-nearest neighbours (k-NN), partial least squares-discriminant analysis (PLS-DA), soft independent modelling by class analogy (SIMCA), classification and regression tress (CART) and random forests with Matlab as data processing software, have been used to determine if an e-liquid contains nicotine or not[67].

Heating propylene glycol can produced the toxic carbonyls formaldehyde (600 °C), acetaldehyde (600 °C), and acrolein (traces at 350 °C)[16]. A free-radical dehydration of glycerol yields 3-hydroxyl-1-propen-1-ol and through tautomerisation 3-hydroxylpropionaldehyde can be formed; the latter one can lose one water molecule through free-radical formation to give rise to acrolein[71]. If the temperature is >400 °C, 3-hydroxylpropionaldehyde can be converted to formaldehyde and acetaldehyde by the retro-Aldol reaction[71]. An interesting study[71] trapped aerosols at different vapour conditions and monitored the formation of aldehyde by means of trapping with 2,4-dinitrophenylhydrazine and assessing by HPLC-UV. The coil

in the electronic compartment will heat the e-liquid when power is applied, this is measured in watts. It is noticeable that different electronic designs produce a different output. While some designs produce a steady increase in the three aldehydes when more power (producing more temperature) is applied, in some other cases the amount remains at a low level and with no increase.

Metal content is a known issue in traditional cigarettes and traces of metals have been found in e-cigarettes. ICP-MS methods have also been used to assess the heavy metal content in e-liquids[72].

4. Health

E-cigarettes are becoming more widely used due to promotion of manufacturers as a healthier alternative to conventional smoking. Amongst the complaints e-cigarette users describe, mouth and throat irritations have a higher incidence, this could be due to carbonyls formed during vaping[16]. It is important to notice that burns are also important consequences from the electronic devices due to faulty or fake batteries and/or mechanisms[73].

Other volatile organic compounds (VOCs) such as toluene and m,p-xylene which can be produced in the process are considered to be carcinogenic, hemotoxic, neurotoxic and irritants[19]. More harmful side effects are continuously being found through *in vitro* and animal models[19,74–78]. The vapour heating process can produce carbonyls, though in not as high concentration as traditional smoking. There is biological evidence that aldehydes are toxic to mammalian cells by acting as mutagens, producing DNA single-strand breaks and chromosomal aberrations[79]. Toxicity comes in different shades, in the case of human and animal subjects, toxicological studies imply the assessment of biomarkers such as pro-inflammatory cytokines, development of cancer, teratogenicity, plasma nicotine concentration and effect on metabolism[80–82]. For animal models a lethal dose can be assessed. In the case of cell culture, toxicity initially appears as cell viability to then follow cell health, metabolic pathways, mutagenicity, release of cytokines and signalling[81,82]. Studies in mice, which are indicators of acute exposure due to high concentrations and short but persistent contact with the vapour, have shown an increase in inflammation markers such as IL-6, IL-1α and IL-13 especially in the lung area[83] and reduction in immune defence towards bacterial and viral infections as the phagocytosis by alveolar macrophages was compromised upon challenge with e-cigarette smoke[84]. In the case of rats[80], e-cigarettes with nicotine affected the body weight and energy intake, alteration in the lipid profiling (though some effect was observed when nicotine was not present). Nevertheless with or without nicotine, the e-cigarettes depleted the hepatic glycogen producing hyperglycaemia and affected the kidneys by altering the anti-oxidant response in both cases with or without nicotine, implying the rest of the ingredients have a toxicological effect on renal ducts[81,82].

Though research on e-cigarette toxicity is not very extensive, the area is not free of controversy as the market is relatively new. Several studies have been carried out on human and animal cells, animal models, stem cells and some short

clinical trials using vapours, smoke extract and similar, obtained from e-cigarette devices as well as the e-liquid refills[83]. There is an increasing amount of research dedicated to the toxicity of the contents of the e-cigarette refills, looking at the biological activity of nicotine, vehicle and flavours[76,83,85]. Studies on human bronchial airway epithelial cells and human foetal lung fibroblast showed that challenge with different flavours of e-liquids[83] exhibited high levels of stress in the form of reactive oxidative species (ROS) and the cell morphology changed to enlarged cells. This is in addition to decreased cell viability, increase of inflammatory markers, and response in neutrophils[21,83,86].

Published research is trying to shed light on the hot topic 'are they toxic' so more studies are focusing on comparing e-cigarettes to tobacco cigarettes. A study comparing both types of smokes on HaCat (non-cancerous human keratynocytes) and A549 found that pro-inflammatory cytokines and chemokines (PDGf-BB, basic FGF, IL-8, IL-12, IL-17, GM-CSF, IP-10, MCP-1, MIP-1β in both cells and IL-1rα, IL-10, G-CSF, IFN-γ, RANTES, TNF-α and VEGF in HaCat) were released upon exposure to e-liquids with cell death more preponderant in the traditional tobacco smoke[87].

A great majority of the biological studies focus on the lung and cardiovascular functions and morphology with the nasal epithelia being overlooked. A study conducted on this topic collected biopsies and fluids from the nasal passage of non-smokers, cigarette and e-cigarette smokers[88]. The changes in the expression of mRNA of keys genes was used to monitor the health of the cells and the metabolic pathways. The findings include a decrease in the expression of immune related genes for electronic and traditional cigarette smokers and in some cases the response was stronger in e-cigarettes, indicating this type of smoking changes the immune composition at the nasal mucosa. A review by Biyani on the area of otorhinolaryngology[89] looked at the implications of e-cigarettes in the paediatric clinic of 80% of adult smokers who started smoking before reaching 18. Though they did not present any clinical trials to determine the effect of passive e-cigarette smoking, they presented the problem of liquid poisoning (as small children believe the bottles to be fruit juices) and young adults start to smoke believing e-cigarettes to be non-toxic.

A decrease in cardiovascular function has been linked to the use of traditional cigarettes, with the main side effect being inflammation, thrombosis and oxidation of low-density lipoprotein that can affect the myocardial activity[4,88,90]. A clinical study sponsored by the Lorillard Tobacco Company, compared traditional cigarettes[91], with both limited exposure (for standardisation, one refill of 16 mg mL^{-1} providing 50 puffs *vs* one Marlboro® Gold King size with both yielding around 0.8 mg, though in real subjects this might vary) and unlimited exposure. Unlimited exposure found the nicotine plasma level to be increased (with the traditional cigarette showing concentrations at 5 min). Also the combination of propylene glycol with glycerine in the e-liquid helps to deliver more nicotine than

propylene glycol alone. The mechanism for heart rate due to nicotine has been elucidated and described by the activation of the sympathetic nervous system with release of norepinephrine and epinephrine upon incorporation of nicotine[92–94]. As the traditional cigarette peaked nicotine in plasma higher and faster than the e-cigarette, the heart rate was increased in correlation to the amount of nicotine in plasma, with this being higher than the traditional cigarette[91]. Though the e-cigarettes have been shown to increase the nicotine content in plasma, affect the systolic and diastolic blood pressure and increase the heart rate, they were under lower values than traditional cigarettes, with them being clinically insignificant at the conditions used. Other studies seem to validate the notion that switching from traditional cigarettes to e-cigarettes (and hopefully then quitting completely) will assist to lower the systolic blood pressure[93,95] and some of them finding the nicotine plasma level to be equal in both e-cigarettes *vs* traditional cigarettes[96].

More recent studies are using different systems to assess toxicity, such as a *C. elegans* model[97], in which refill components (nicotine, propylene glycol and flavourings) were tested; oxidative stress, growth and brood size were affected the same way when tested as both liquids and vapours.

It is important to note that many of the studies arrive at the same conclusion regarding the biological activity as well as the analytical composition, but parallels are difficult to be drawn amongst the many different studies as concentration of the dosing sample varies as well as conditions (such as feeding media, time and type of exposure) presented in the literature. An excellent review published in ATLAS[98] comments on the majority of the *in vitro* methods used (2-dimensional, 3-dimensional) and different types of assays to study toxicology, risk assessment, cell transformation and cell health assays and genomic analysis of tobacco products; they could be extremely important when planning biological research.

5. Marketing and metrics

With the world-wide market reaching over £35 billion by 2025[7,8] not much emphasis can be found on the marketing e-cigarettes receive, but a presentation by Monks and Crawford (Texas Tech University Health Sciences Center, El Paso) obtained on the USA Environmental Protection Agency website[73], provides some interesting numbers. In the UK, figures obtained from Action on Smoking and Health (ASH) showed e-cigarette user numbers rose from 700,000 in 2012 to 2.1 million by 2014[8]. For 2014, in the USA, around 3.9% of this population (450,000 early teens) smoke e-cigarettes, with 13.4% (2 million) teens smoking them. This highlights the growing tendency of this habit; on the other hand this teen population fell from 15.8% of tobacco smokers to 9.2% by the same year[7,8]. As this overall population is under 18, and banning laws apply, more disguises (Tic Tac boxes, juice bottles) are found for e-cigarettes to be smoked. Calls related to e-cigarette poisoning in the state of Texas showed that 57% was related to children younger than five years old. They were unintentional with 96% in their houses, 85% from ingestion and 11% dermal. This is an important aspect as the marketing directed at adults is

also affecting small children. The e-cigarette marketing has been very aggressive, many of them with a strong sexual content, or trying to relate to foods/diets or traditional cigarettes as well as using celebrities; for this the advertising expenses have increased from $6.4 million in 2011 to a staggering $112.9 million in 2014[73].

In this review, we screened the web for information on the composition and toxicity of e-cigarettes, with an emphasis on flavour activities and health profiles. Analytical studies have shed light in the complex composition of e-cigarettes, in which flavouring additives had an unknown effect on the delicate lung epithelia.

6. Case study

We present a case study, in which we used LCMSMS to assess the analytical content of a group of e-cigarette refills and compared our results with published data. Not surprisingly, our results are in agreement with other groups while using similar analytical techniques. In this case, we also studied the biological activity of e-cigarette refill ingredients in a normal cell line representative of human lung epithelia. To make the case study more relevant to the wide variety available, we selected different branded e-cigarettes with different flavours, with and without nicotine. In accordance with the majority of the published research available, we also found the refills to be toxic to the cells, with some being less toxic and some allowing the cells to recover after the challenge. For this case study, we purchased around 18 samples of e-liquids and challenged the human bronchial cell line Beas2B to different concentration of the e-liquids from 1 day to 3 days of exposure.

6.1. Materials and methods

6.1.1 Materials

Thermo Fisher Scientific, Altrincham, Greater Manchester, UK: 0.25% Trypsin-EDTA (GIBCO), sterile phosphate buffered saline, acetonitrile optimal LCMS grade (ACN), ammonium acetate analytical grade, formic acid optimal LCMS grade. Lonza, Slough, Berkshire, UK: BEGM Single-Quot kit. Sigma-Aldrich, Dorset UK: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), sterile phosphate saline buffer (sPBS), nicotine, propylene glycol, dimethyl sulphoxide (DMSO), chlorpromazine. ECACC, Porton Down, Salisbury, UK: Beas2B cell line (immortalised cells obtained from autopsy of normal human bronchial epithelia from non-cancerous patients). VWR West Sussex, UK: plastic ware. Anachem, Luton, UK: pipettes and pipette tips. Amazon UK: e-cigarette refills. Superdrug, Manchester, UK: Nicolite refills. Hichrom, Reading, Berkshire, UK: 0.2 μm polypropylene syringe filters.

6.1.2 Cell maintenance

Cells were grown as adherent monolayer culture in 75 cm^2 flasks in Bronchial Epithelial Growth Medium (BEGM) using Lonza's BEGM SingleQuot kit, at 37 °C,

and under a humidified atmosphere containing 5% CO_2 and 95% air. Cells were passaged twice a week after reaching 70% confluence.

6.1.3 MTT assay

Two types of e-liquids are available on the market, synthetic, containing artificial flavours, and natural, containing extracts of tobacco leaves and natural flavours extracted from plants. Pre-packed cartridges can have varying nicotine concentrations (ranging between 0–18 mg mL^{-1} nicotine/cartridge) with diverse flavourings for example tobacco, menthol, mint, chocolate, apple, cherry, caramel and many more[12,13]. We used different suppliers that were commercially available over the counter and through the internet. We tested a variety of flavours and nicotine content; as well as synthetic nicotine and propylene glycol which is used generally as carriers for the production of the vapours. All e-cigarette refills, nicotine and propylene glycol, were tested at a range of concentrations (0, 4, 10, 20, 40, 80, 120, 160 and 200% puff, with each puff being 5 µL [100%]) for e-cigarettes with dilutions in sterile water, 0–1.64 mg mL^{-1} for nicotine stock, 0–0.1 g mL^{-1} for propylene glycol stock and 0–100 µM for chlorpromazine (this is the positive control in all assays). Cell death percentage was determined by the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] micro-culture assay. Cells were detached from the 75 cm^2 flasks (at a confluence of 70%) by trypsinisation, seeded in 100 µL aliquots into 96-well clear micro-culture plates. Cell densities were 40,000, 30,000 and 20,000 cells mL^{-1} for 24, 48 and 72 h of incubation, respectively. This method was chosen in order to ensure exponential growth of untreated controls throughout the experiment. Cells were allowed to adhere into the 96-well micro-culture plate for 24 h prior to dosing. Stock solutions of the test compounds in water were appropriately diluted in complete culture media to make up the required concentrations, and then added in 10 µL aliquots into the 96-well micro-culture plate. Cells were exposed to the test compounds for 72 h. Plates were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. At the end of the incubation period 30 µL $well^{-1}$ MTT solution in sPBS (3 mg mL^{-1}) were added, then incubated for a further 3 h. After the end of the incubation, the supernatants containing medium and MTT were removed and the formazan crystals formed by viable cells which were dissolved in 100 µL of DMSO per well. Optical densities at λ=540 nm were measured with LUMIistar Omega multi-mode plate reader (Edinburgh, UK). The colorimetric MTT assay was used to determine the cell death percentage at serial diluted concentration of the tested compounds and the concentration at which 50% of cell growth was inhibited (IC_{50}), as compared to the control wells which did not contain any test component as determined from a dose–response curve using OriginPro 9.1 (Northampton, MA, USA) data analysis and graphing software. Chlorpromazine was used as a positive control in the MTT assay. Data were collected as duplicates and statistical analysis calculated as standard deviation (SD) using Excel Microsoft (Reading, Berkshire, UK). Pictures were taken with a microscope Axio Vert.A1, PE-300 from Zeiss, Cambridge, Cambridgeshire, UK.

6.1.4 Mass spectrometry

The analysis was performed on Agilent 6540 LCMS-MS Q-ToF Jet Stream ESI (Greater Manchester, UK). Conditions: +2500 V, CE (collision energy) 80 eV, sheaf gas 350 °C at 10 L min^{-1}, drying gas at 325 °C at 10 L min^{-1}. Nebuliser gas pressure was at 18 psi. The chromatography was performed on an Agilent 1260 series (Greater Manchester, UK) with auto sampler and thermal controlled column chamber. The separation was done on a Thermo Scientific Accucore HILIC 50×2.1 mm particle size 2.6 µm (Thermo Fisher Scientific, Altrincham, Greater Manchester, UK) kept at a stable 20 °C. The flow rate was set at 0.4 mL min^{-1} using a gradient profile of ACN (acetonitrile) 95%:H_2O 5% (0.1% formic acid/5 mM ammonium acetate) 0 min: 100%. 20 min: 60%. 25 min: 100% end 30 min, with the remainder being H_2O (0.1% formic acid/5 mM ammonium acetate). The column was prepared and stabilised for 6 h before running using ACN 95%:H_2O 5% (0.1% formic acid/5 mM ammonium acetate with a flow rate of 1.0 mL min^{-1}. The samples were prepared by diluting the e-cigarette fluid 10 µL with 990 µL of ACN and filtered through 0.2 µm polypropylene syringe filters (this is also known as 'dilute and shot methodology').

6.2. Results and discussion

We tested 18 different e-cigarette refill flavours for their toxicity on human derived bronchial cells (Beas2B). This case study aimed to confirm the literature results by exposing the cells at different concentrations and times. The definition of 'puff', its volume and quantity seems to be different in various publications, as made with reference to the total reservoir and expressing it into nicotine content[4,14,99,100]. The puff is also dependant on the user, with some puffing a larger volume than others. Based on literature evidence of amount of nicotine used[14,99,100] and the given value per cartridge of nicotine at an average of 18 mg mL^{-1}, we calculated that one inhalation might be equal to 5 µL, and this puff would contain around 90 µg of nicotine.

6.2.1 Biological data

In this work, we will equate one puff to 5 µL of the e-liquid refill. The Beas2B were exposed at 0, 4, 10, 20, 40, 80, 120, 160 and 200% puffs. This in effect means 0, 0.2, 0.5, 1, 2, 4, 6, 8, 10 µL of refill respectively (dilutions were made with distilled sterile water). Exposure was for 24, 48 and 72 h. The bronchus conducts the air into the lungs in which the surface area[44] can vary in the range of 40–80 m^2. Although one puff (5 µL) appears to be a large volume, smokers rarely would have only one puff. Instead there would be a continuous flow; different groups might have a diversity of approaches to quantify the puff, nevertheless the main agreement seems to be centred on the amount of nicotine delivered.

In Table 1, we show the IC_{50} values obtained of duplicate results. At 24 h, the IC_{50} values ranged from 1.12 to 70%, making some e-cigarettes based on menthol, tobacco and butterscotch flavours the most toxic (Figure 1). The same pattern seems to be repeating itself at 48 and 72 h with ranges between 6.3–40% and 1–92% respectively. Propylene glycol seems to show more toxicity when the cells were

Table 1 *IC_{50} values of different e-cigarette refills tested on Beas2B at 24, 48 and 72 h (results for duplicate determinations)*

IC_{50} (% of puffs, ±SD)	24 h	48 h	72 h
Aulola Butterscotch*	7.4±5.2	10.1± 3.6	9.2±1.7
Vapouriz Bubblegum*	28.3±0.6	26.3±10.5	17.1±2.1
Vapouriz Vanilla Velvet*	25.5±1.6	19.3±0.9	79.0±0.3
Vapouriz Banana*	12.5±1.7	39.8±0.4	26.0±0.9
Vapouriz Grape*	32.7±5.8	29.7±9.7	91.6±0.2
Dekang CherryBlossom 18 mg mL^{-1}	20.9±4.0	22.8±1.8	24.7±0.8
Vapouriz Blueberry*	28.5±1.8	12.3±3.8	30.1±8.8
Dekang Blueberry Mist*	37.6±0.1	24.5±0.1	21.0±1.1
Vapouriz Strawberry Bliss*	20.8±0.3	17.3±0.1	18.2±0.6
Vapouriz Juicy Apple*	29.8±6.3	21.5±3.5	28.7±0.6
Nicolite Menthol 16 mg mL^{-1}	8.7±0.9	6.3±2.6	2.8±14.4
Dekang Menthol 18 mg mL^{-1}	21.7±0.6	10.8±2.4	12.3±1.2
Vapouriz Menthol Special blend	1.1±1.6	18.4±1.7	22.2±4.1
Vapouriz Icemint	68.9±0.1	32.9±0.9	19.3±0.8
Vapouriz Classic Tobacco	4.3±7.3	9.5±3.9	11.9±6.5
Vapouriz Virgin Tobacco	30.9±5.8	15.2±0.8	<1±2.19
Nicolite Tobacco 11 mg mL^{-1}	21.9±1.2	36.9±0.3	11.5±2.5
Nicolite Tobacco 16 mg mL^{-1}	31.0±3.2	11.9±14.7	24.7±7.6
Nicotine stock 18 mg mL^{-1}	32.3±1.5	27.2±1.8	13.9±2.9
Propylene glycol stock 1 g mL^{-1}	14.7±5.4	9.5±19.2	5.7±2.2
Chlorpromazine[1]	3.1±9.0	1.5±4. 7	1.0±0.2

*These samples do not contain nicotine.
[1]IC_{50} in μM, ±SD.

exposed for longer times (48 and 72 h), while nicotine was quite consistent through the total study. Flavours like grape, blueberry, cherry and some menthol blends produced the lowest toxicity for the brands tested.

To have a clearer understanding of the results, we plotted the IC_{50} values (Figure 1). It is interesting to note that at 24 h the majority of the samples tested are very toxic with the IC_{50} values in the lower 1/3 band on the y axis. At 48 h this tendency changes to be more in the middle band and at 72 hours there is a clear tendency for higher toxicity (lower band in the y axis). It is interesting to note the e-cigarettes flavoured of vanilla and grape are the least toxic samples. Icemint can be a complex mixture and this can be observed in the increasing toxicity it showed on the cells.

Pictures of each sample at dosages of 200, 120, 10, 4 and 0% puffs (a summary in Figure 2 and the remaining pictures and IC_{50} curves in the Appendix) were taken. In Figure 2, the pictures show the cells exposed to a low puff concentration (10% of puff) for the longest period of time (72 h).

In our set of 18 e-cigarette refill samples, we studied different fruit flavours as well as candy flavours such as butterscotch and bubblegum. The butterscotch flavour when inhaled has been found to be responsible for a particular lung condition in employees working in popcorn factories, called popcorn lung syndrome[101].

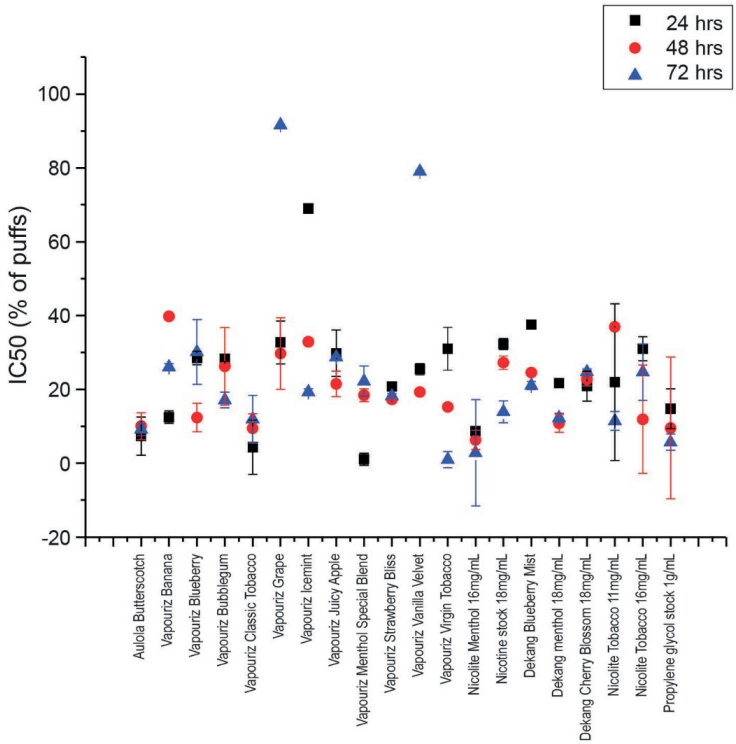

Figure 1 *IC_{50} values at 24, 48 and 72 h.*

Exposure to diacetyl in the working environment affects the middle and lower airways producing a cough, dyspnoea, and *bronchiolitis obliterans*[102]. This flavour has been discontinued in the market of electronic cigarettes, even though reports[7] express concerns as this is found in around 75% of all e-liquid samples. Alternatives have been proposed (such as 2,3-pentadione, 2,3-hexanedione, 3,4-hexanedione and 2,3-heptanedione) and studied on murine models with results indicating they might not be completely safe[103]. The sample we obtained was shown to be very toxic, with IC_{50} values for 1, 2 and 3 days of exposure around the value of 10% of a puff (0.5 µL). The pictures clearly show how cell numbers are low and the cells are very elongated when compared to cells exposed to the media only. The other candy flavour, bubblegum, though toxic, had IC_{50} values in the range of 20 to 30% of a puff, with cell numbers higher and a slightly rounder shape.

Vanilla is a popular flavour in sweets. We tested a vanilla refill, and we found the IC_{50} values to be moderately toxic at 24 and 48 h of exposure (~20%) and much less toxic at 72 h (80%). This implies that the cells can recover with time if not exposed continuously, an indication of the possible health benefits of e-cigarettes. The cell numbers in the photographs (Appendix) not only showed higher survival rates, but the cells were forming islands which is characteristic of lung type cells. Menthol and mint are very popular flavours, so we tested one sample of mint (icemint) and three samples of menthol. We found that the mint flavour was less toxic after one day

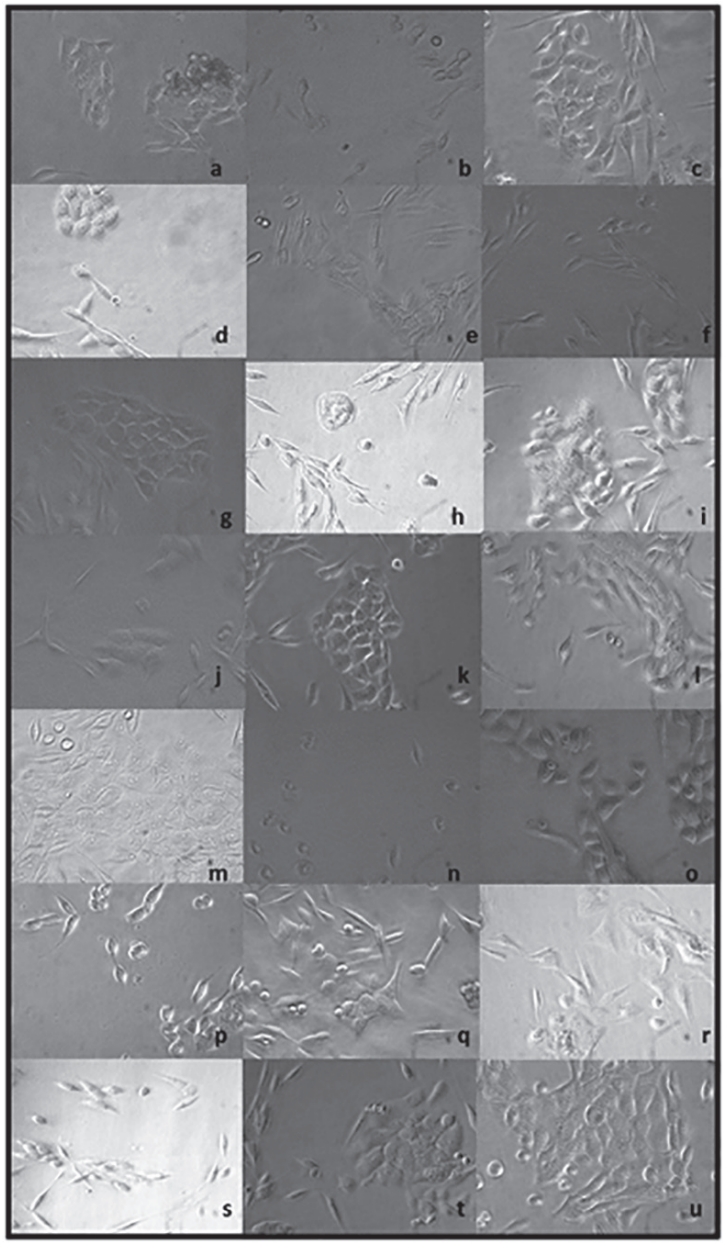

Figure 2 *Cells exposed to 10% (0.1) puff for 72 h: (a) Aureola Butterscotch; (b) Vapouriz Banana; (c) Dekang Blueberry Mist; (d) Vapouriz Juicy Apple; (e) Vapouriz Bubblegum; (f) Vapouriz Grape; (g) Vapouriz Strawberry Bliss; (h) Nicolite Menthol 16 mg* mL^{-1}*; (i) Vapouriz Vanilla Velvet; (j) Dekang Cherry Blossom; (k) Vapouriz Blueberry; (l) Dekang Menthol; (m) Nicolite Tobacco 16 mg* mL^{-1}*; (n) Vapouriz Virgin Tobacco; (o) Nicolite Tobacco 11 mg* mL^{-1}*; (p) Vapouriz Classic Tobacco; (q) Vapouriz Icemint; (r) Vapouriz Menthol Special Blend; (s) nicotine stock (pharmaceutical grade) (0.08 mg* mL^{-1}*); (t) propylene glycol stock (0.005 g* mL^{-1}*); and (u) media.*

of exposure (IC_{50}=70%), but became more toxic the longer the cells were exposed (IC_{50} in the range of 30–20%). Furthermore, the cells also looked very unhealthy after the initial times of exposure, but showed remarkable recovery towards the 72 h period of incubation. Possibilities could include the cells managed to metabolise the toxic contents to less damaging agents. The menthol samples were in general very toxic with IC_{50} values for all incubation times lower than 20%. The cells looked to be elongated and in the majority of cases quite isolated. It is interesting to notice that samples from different suppliers have different toxicity, giving rise to the question what the ingredients are or at least the percentages in these refills.

E-cigarettes, as a relatively healthier option, have much less ingredients than a tobacco based cigarette, and do not reach the extremely high temperatures of combustion. Nevertheless, because the tobacco flavoured e-cigarette is popular amongst consumers, we tested four samples of tobacco based e-cigarettes with different concentrations of nicotine. We found them all to be quite toxic, for example the classic tobacco flavour has IC_{50} values around 10%, and for tobacco with nicotine it was around 10–30% in a sample of virgin tobacco. It also became very toxic the longer the cells were exposed to the liquid. The cells looked much damaged at high concentrations and short exposure times, becoming very flat and elongated towards the end of the experiment.

Fruit based flavours are very popular with younger generations. Suppliers might use natural or synthetic flavours to produce the desired flavour. From all the samples tested, except Dekang Cherry Blossom, they did not have nicotine in the ingredients list. This could explain why in general these samples were less toxic. We tested refills of flavours of banana, blueberry, grape, apple, strawberry and cherry. We found them to be moderately toxic (IC_{50} values in the vicinity of 30%), with the grape flavour being the least toxic one. Nevertheless, at high concentrations of refill liquid and short exposure times the cells look disperse, elongated and damaged. Towards the end of the trial, the cells looked healthier, forming some islands, thus showing better recovery.

We tested a stock of nicotine and the propylene glycol carrier control. We found nicotine to be moderately toxic in the range of what it would be expected to appear in puffs (IC_{50} values between 15–30%). We also found that propylene glycol became increasingly toxic the higher the volumes of the puff and the longer the exposure times were (IC_{50} values between 5–15%). In both cases, cells looked unhealthy with a tendency to recover.

Interestingly a study performed on HaCat (normal human immortal keratinocytes), HN30 (human neck squamous cell carcinoma from a primary laryngeal tumour) and UMSCC10B (human neck squamous cell carcinoma from a metastatic lymph node) for which vapour of e-cigarettes with and without nicotine were tested found ~1.5 fold for samples without nicotine and up to 3-fold for samples with nicotine when DNA strand breaks were tested and increased cell death[104]. Extrapolating the results from *in vitro* to *in vivo* does not seem to be an easy subject, as there are many variabilities in the e-cigarette delivery due to different electronic

devices, and some research points in the direction of the nicotyrine hypotheses[105]. This chemical product of the oxidation of nicotine seems to accumulate in e-liquids with time when it is exposed to air. It is a reversible inhibitor for CYP2A13 in the nasal and respiratory epithelia, and irreversible inhibitor of CYP2A6 in the liver. The hypotheses postulates that nicotine is delivered more effectively if nicotyrine is present as it facilitates the absorption in the airway epithelia (by inhibiting CYP2A13) and inhibiting nicotine's metabolism in the liver (by inhibiting CYP2A6), therefore raising the nicotine's plasma concentration and hence relieving the nicotine craving[105]. Though data seems to support it, more evidence needs to be acquired and a more complex approach needs to be taken which might not be able to be studied at a single cell level.

6.2.2 Analytical data

We studied the 18 samples for their composition as well as nicotine content. Using state-of-the-art mass spectrometry equipment, we developed new liquid-chromatography methodologies to test the ingredients and analyse the content of the main toxicant. An Accucore HILIC column was employed, and in it, nicotine showed a retention time of 7.55 min. The sample was measured using MS/MS fragmentation 163.1230 *m/z* → 131.0650 *m/z* with CE of 30 eV and a dilution curve with a highest amount of 25 mg mL^{-1} was prepared to quantify the areas related to the nicotine content. The results (Figure 3) shown for the samples which according to the manufacturer's labels should be nicotine free, had quantifiable levels of nicotine within them, the majority of them had extremely low amounts in the low ppm (part per million) though some, such as butterscotch, had 0.015% and juicy apple with 0.03%. On the other hand, the levels of nicotine in samples in which have stated nicotine content (manufacturer's labels), vary depending upon the producer (Figure 4). The Nicolite brand, showed a large variation in the analysed to stated amounts. The 11 mg mL^{-1} sample showed to be closer to 9 mg mL^{-1}, and the 16 mg mL^{-1} samples that had different flavours such as tobacco and menthol, ranged from almost 6 mg mL^{-1} to almost 12 mg mL^{-1}. The other manufacturers, Dekang and Vapouriz, for which the labels described a content of 18 mg mL^{-1}, showed a range of 16 mg mL^{-1} to 18 mg mL^{-1}. The levels of nicotine are likely indicators that GMP (good manufacturing practices) are not being followed by some manufactures of the e-cigarette fluids, and may run afoul of the current manufacturing guidelines set by the European Union[106]. It is interesting to note that nicotine based e-cigarette refills showed the highest toxicity with IC_{50} values ranging from 3 to 25% puff for the 72 h period of incubation on the Beas2B cells.

One of the most concerning flavour ingredient in e-fluids is the butterscotch flavouring, diacetyl flavouring (a diketone), (1.2 min retention time) which is known to produce lung disease when inhaled[101,106,107]. We analysed the AV Butterscotch flavour e-cigarette refills, and we found the content of diacetyl (presented a retention time of 1.2 min) was 10.625 molecular count which in real terms means traces. This comes as good news for e-cigarette smokers, as other flavourings can be used to

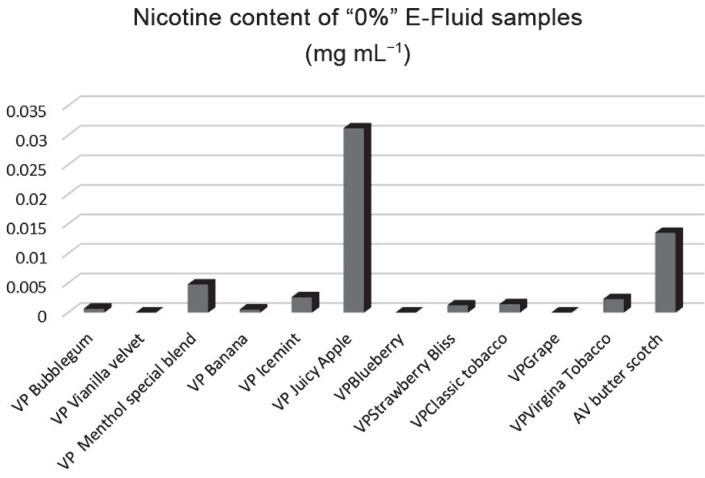

Figure 3 *Analytical determination of nicotine in the e-cigarette refills for which the nicotine content is 0 (zero) according to the package information.*

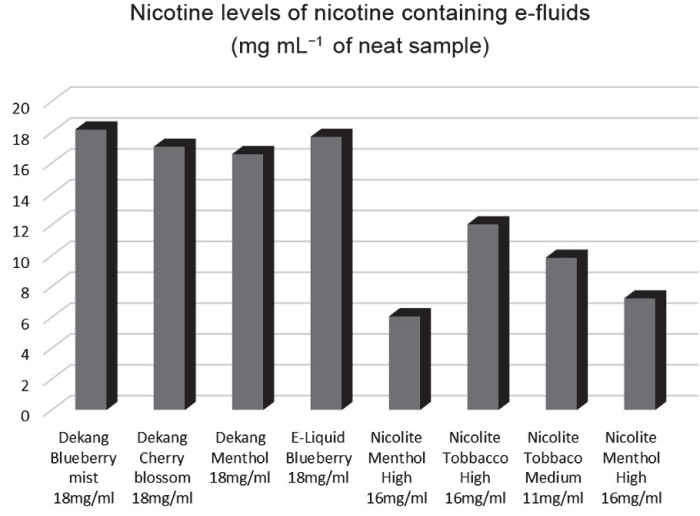

Figure 4 *Analytical determination of nicotine in the e-cigarette refills for which the nicotine content varies from 11 mg mL^{-1} to 18 mg mL^{-1} according to the package information.*

mimic the butterscotch flavour or aroma. Nevertheless in this particular e-cigarette refill sample, the biological data showed high toxicity in the biological assessment, implying the flavouring agent (possibly another member of the diketones family) is also toxic[108].

Tobacco flavours are extremely popular as it might give the e-cigarette smoker the sensation of a real cigarette but without the toxins. We investigated four samples of refills containing tobacco flavour and we found (in Figure 5 the results are presented as molecular counts and they are actually traces in the low ppm of flavours only) they contain traces of several other chemicals including flavouring agents such

as vanillin, ethyl butyrate (tropical flavour), ethyl vanillin, ethyl-methyl-maleimide (tobacco), β-damascone (fruit), butanedione (butter) and benzyl alcohol (fruit). Investigation of the flavour profile of the tobacco flavoured e-fluids showed that it is possible to 'fingerprint' the different manufacturer batches. While the number investigated was small it does open up the possibility of a database for forensic analysis of e-cigarettes. Overall we found the e-cigarette refills contain around 99% of the carrier, with this being generally propylene glycol, up to 0.8% of nicotine (near 0% in the free nicotine refills), 0.018% sweetener (in the form of maltol/ethyl maltol and other sweetening flavours) and 0.002% of flavouring agents, including unknowns. It is this 0.002% that would help to fingerprint a sample. Our data is quite in agreement with other studies[63] that have reported e-cigarette liquid to contain glycerol or propylene glycol (≥75%), water (≤18%), nicotine (~2%) and flavours (~10%). This case study demonstrates that different approaches can reach similar conclusions, though the majority of the published research employs GCMS, the use of LCMS is equally valid.

In our data, we also found all the samples from the brand Vapouriz to contain dodemorph (as $[M+H]^+$=282.2779), which is an antifungal[109], all Dekang samples had the alkaloid sauroxine (as $[M+H]^+$=275.2108)[110], all Nicolite samples contain the alkaloid cytisine (as $[M+H]^+$=191.1178)[111] and all samples containing nicotine also presented the aromatic amine 1-naphthylamine (as $[M+H]^+$=161.1069)[112]. From them only the latter one, 1-naphthylamine, has been found in traces in cigarette smoke[113]. Though it is uncertain of the biological effect of these individual chemicals on the cells, we could not find a correlation between brands with or without the compounds.

All the tobacco samples proved to be highly toxic with the Vapouriz ones presenting the highest cell death rate. Incidentally they have less ethyl-methyl-maleimide though they have higher levels of vanillin based flavour. The

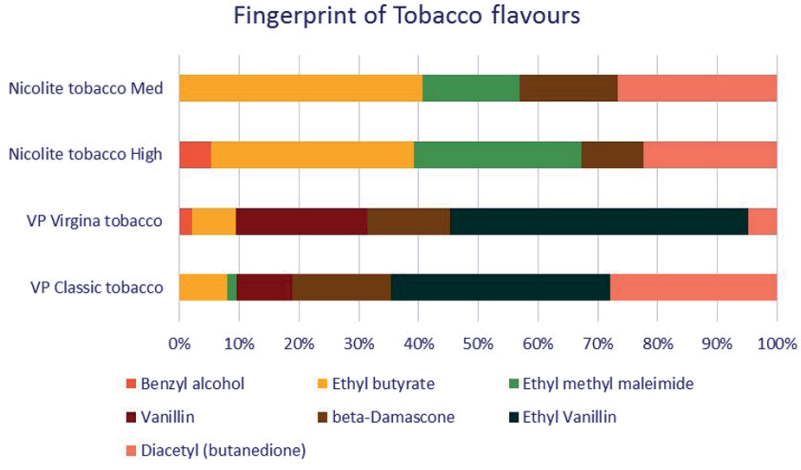

Figure 5 *Analytical determination and fingerprint analysis of tobacco flavour in the e-cigarette refills.*

small difference in the tobacco samples for the IC_{50} could be due to unknown ingredients in the refill, as many times manufacturers use natural or complex extras.

In this case study, we confirm the analytical composition and biological activity of a group of commercially available e-cigarettes. By using LCMSMS we introduce another example of the benefits of employing this type of methodology, unexpectedly we also detected compounds that have been described before (dodemorph, sauroxine and cytisine) suggesting that more research is needed to elucidate the complex compositions. Cell viability is the method of excellence to screen, in a short time, how toxic a compound is, and the majority of the biological data published on e-cigarettes have used the same technique but on different types of cells. In this review, we compared several biological matrices and we confirmed in this case study that e-cigarette refills are poisonous, some moderately and some highly toxic to lung cells.

7. Conclusion

Research by Action on Smoking and Health (ASH)[1] showed e-cigarette use has rapidly increased[114] with the teenage group increasing 800%[18], and e-cigarettes are considered as one of the options helping people to quit smoking. However, the safety and reliability of e-cigarettes has to be reviewed extensively. Much of the literature reviewed infer that e-cigarettes are not free of emission[14–16,33,42,85,95,115] as they release an aerosol containing acetaldehyde, formaldehyde, nicotine, propylene glycol, glycerol and flavourings, with users and those who are exposed to second-hand inhalation being affected[19,75]. Our work supports the opinion that e-cigarettes and especially the ingredients of the e-liquid, which can change in structure after the process of heating, have not been thoroughly characterised or evaluated for safety[76]. The evaluation of the results of this investigation supports our hypothesis that certain flavours of e-liquids, like menthol, tobacco and coffee are more toxic than others such as banana or apple, which shows less toxicity on Beas2B cells by direct liquid exposure.

In a previous study, the cytotoxicity e-cigarette refill samples using human embryonic and adult cells showed that the majority of samples were moderately to highly toxic to the embryonic cells, but less toxic on the adult cells. Also, the cytotoxicity was correlated to the other components of the fluids rather than the presence of nicotine[76]. In another study, the cytotoxicity of liquid (smoke) flavourings was assessed and compared with that of cigarette smoke condensate. They found that cigarette smoke condensate was generally less toxic than liquid smoke flavourings on Chinese Hamster Ovary cells (CHO)[77]. Published results have shown in *in vitro* studies that human bronchial cells exposed to different e-cigarette vapours had mutations in the gene patterns similar to exposure to tobacco smoke[116].

The existing research does not indicate e-cigarettes are completely safe, even though the delivery of nicotine without the toxins found in tobacco cigarettes makes them a safer option. E-cigarette vaping is less toxic than smoking normal cigarettes,

and this group of users benefit from this new technology. Nevertheless e-cigarettes contain toxicants including nicotine, flavourings and volatile compounds, and their thermal degradation products.

We have clearly shown that flavours such as menthol, tobacco, and butterscotch can be considered toxic. However, the assumption that e-liquids with nicotine, especially with higher concentrations of 16 mg mL^{-1}, could be more toxic than the one without nicotine, could not be proven. This could be due to the complex mixtures of solvents, flavouring agents, sweeteners, enhancers and preserves. Nevertheless, e-liquids such as blueberry and tobacco are more toxic with a lower IC_{50}-value then e-liquids with nicotine.

Public Health England (PHE) has endorsed the use of e-cigarettes to help smokers to quit the habit[117]. Evidence seems to indicate smoking e-cigarettes is healthier than traditional tobacco cigarettes so for the traditional cigarette smoker this is a good option, especially if it allows quitting overall. But concerns have been raised for the passive smoker and the younger generations who find smoking e-cigarettes an exciting new habit[24,73]. Politics, policies and funding seem to play an important role in the evaluation of the safety of e-cigarettes and more independent, long-term research needs to be obtained to determine how safe e-cigarettes really are[24].

The work reported in this review alongside a case study further contributes useful and new information to the debate on the safety of e-cigarettes and the different flavouring liquids consumed by users in the devices, and clearly indicate some areas of concern which warrant closer attention in future. This is in agreement with a recent clinical trial in which several toxicant biomarkers (nicotine and metabolites) from both traditional and e-cigarettes were monitored and it was shown that exposure was reduced upon switching[17,18].

8. Acknowledgements

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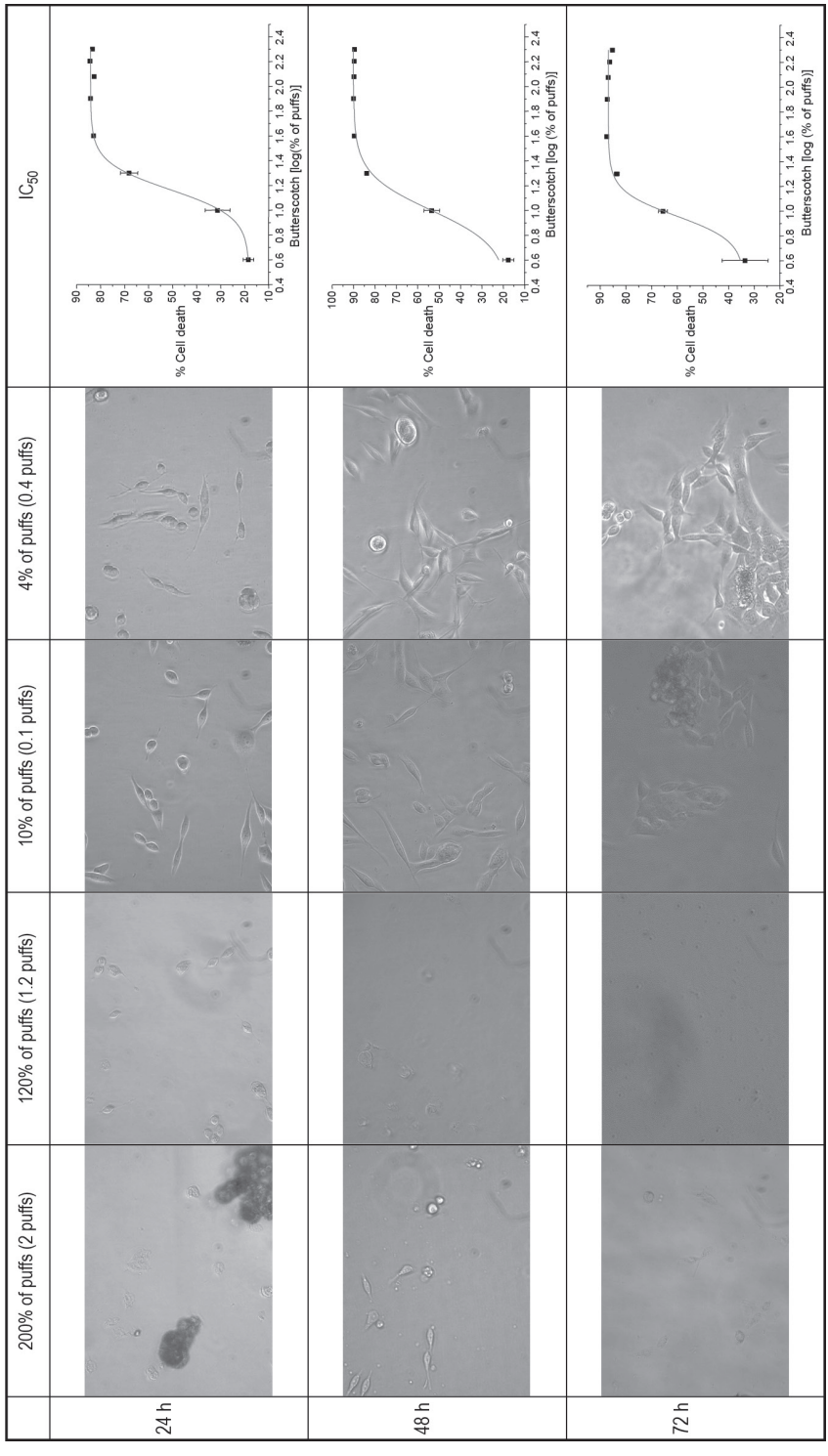
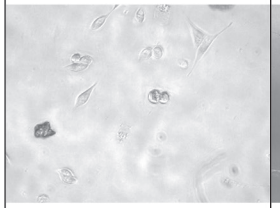
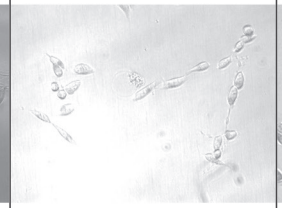
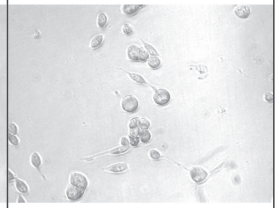
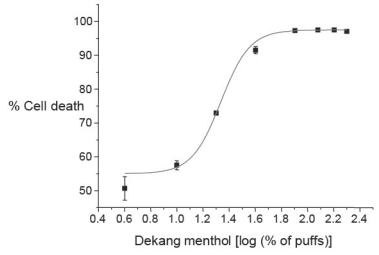
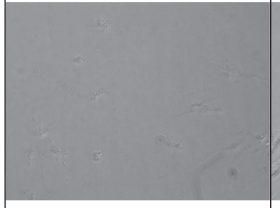
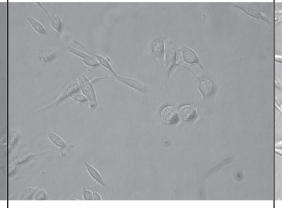
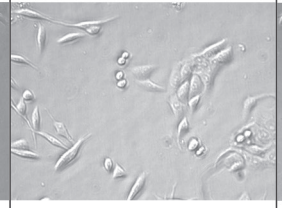
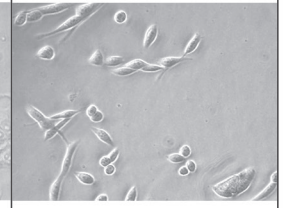
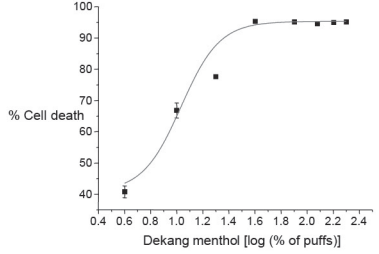
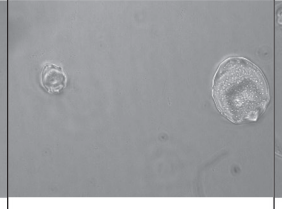
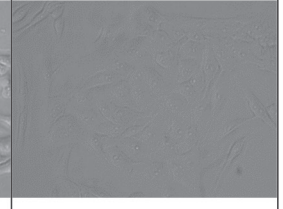
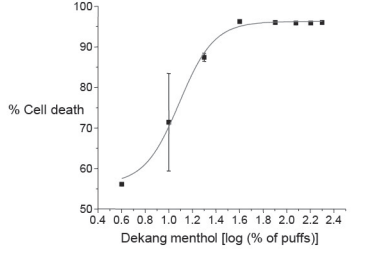
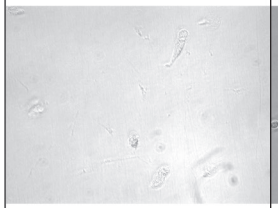
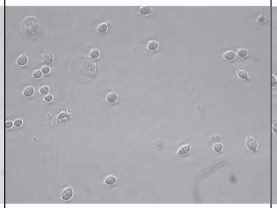
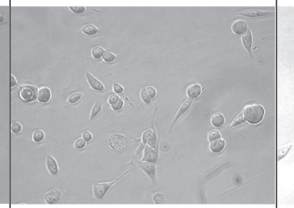
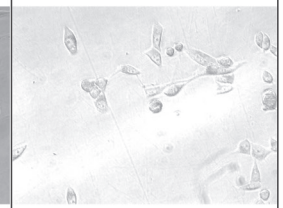
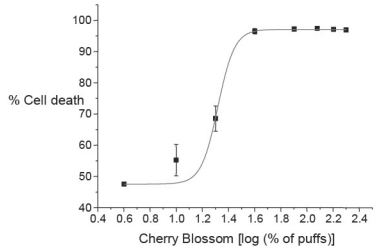
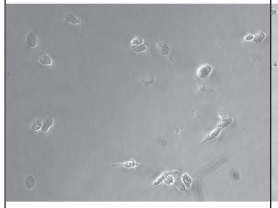
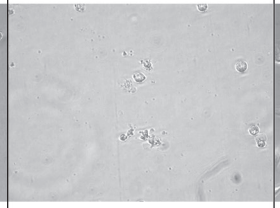
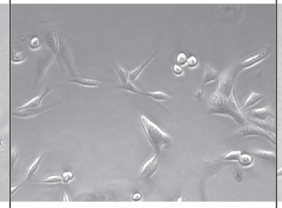
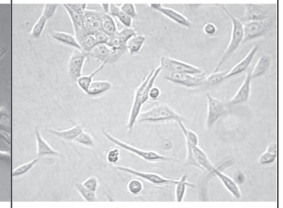
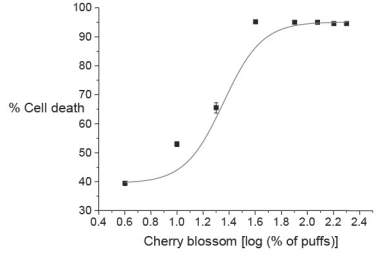
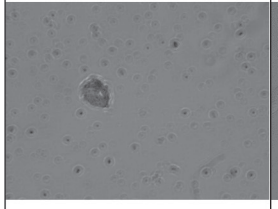
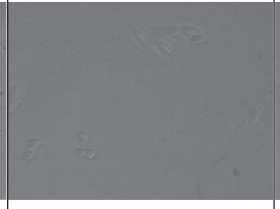
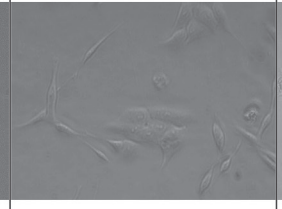
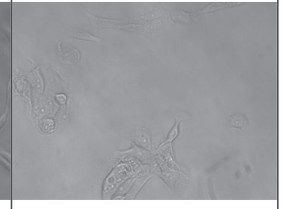
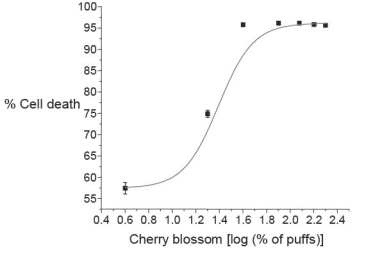
Appendix


Aulola Butterscotch
200% of puffs (2 puffs)
120% of puffs (1.2 puffs)
10% of puffs (0.1 puffs)
4% of puffs (0.4 puffs)
IC50
24 h
48 h
72 h
% Cell death
Butterscotch [log(% of puffs)]
Butterscotch [log (% of puffs)]

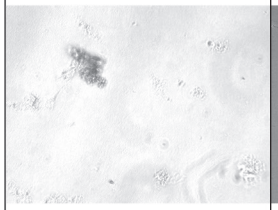
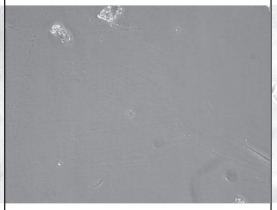
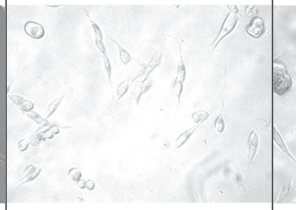
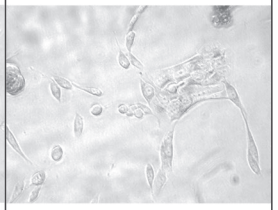
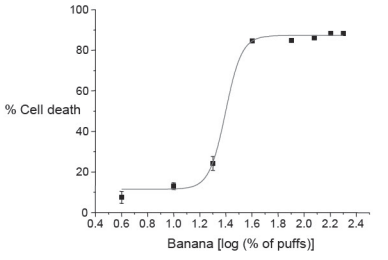
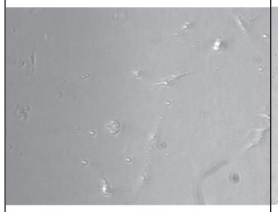
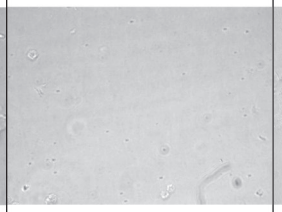
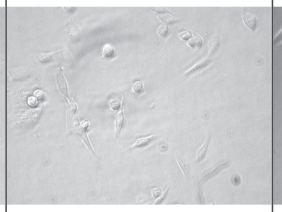
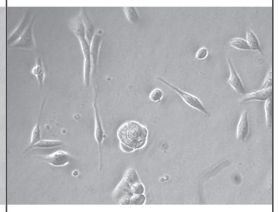
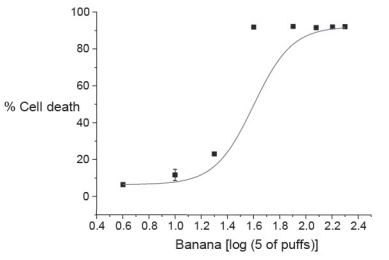
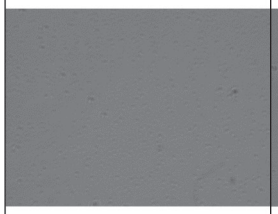
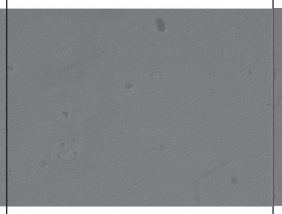
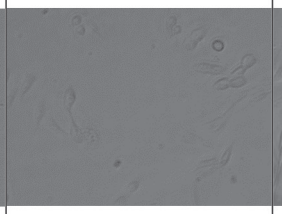
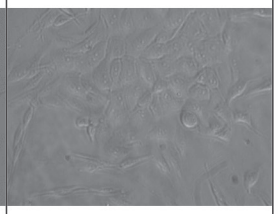
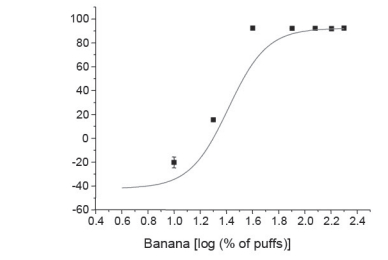
Dekang Menthol

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

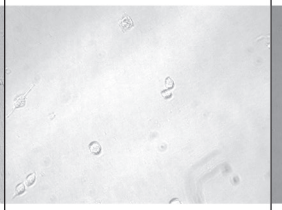
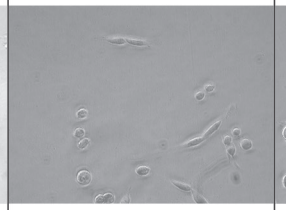
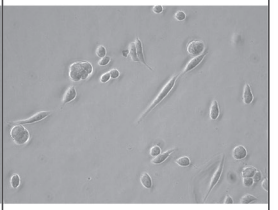
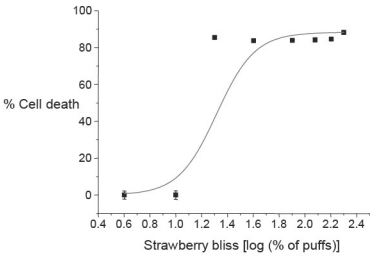
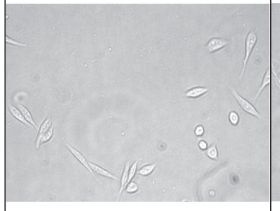
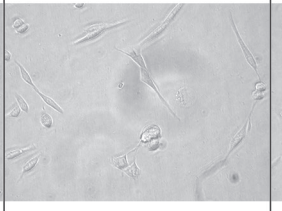
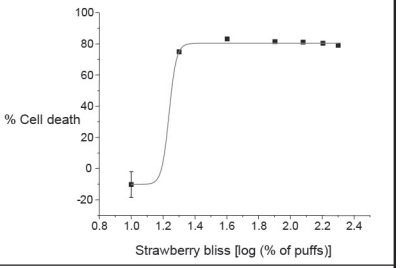
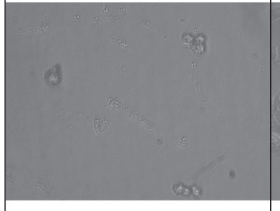
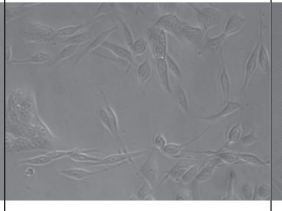
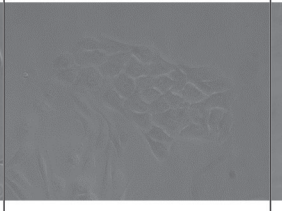
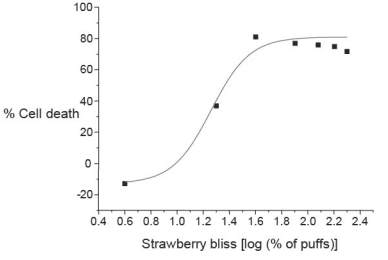
Dekang Cherry Blossom

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

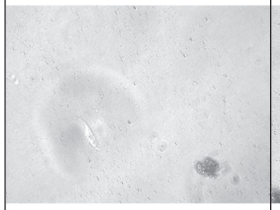
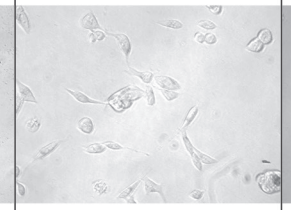
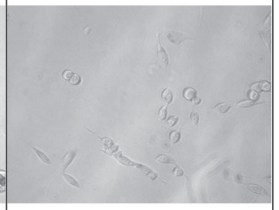
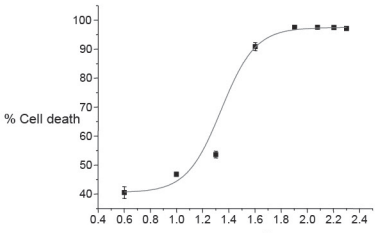
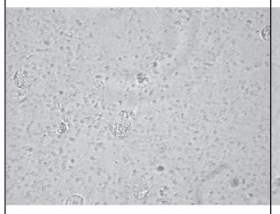
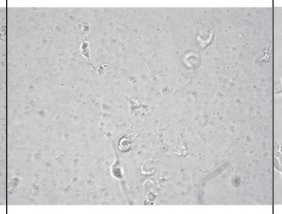
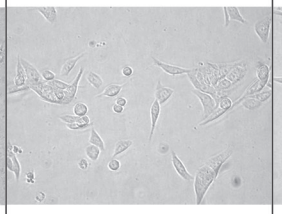
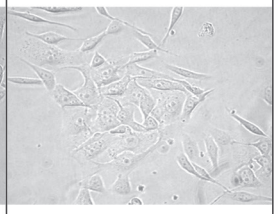
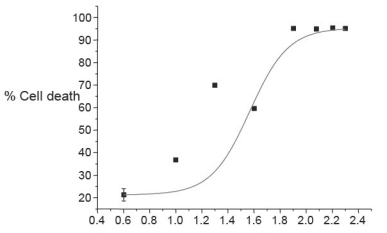
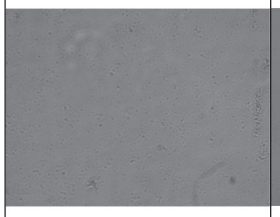
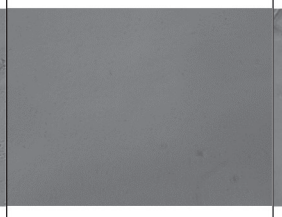
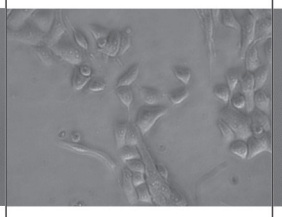
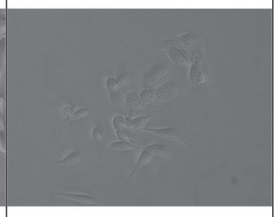
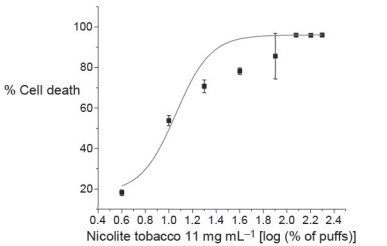
Vapouriz Banana

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

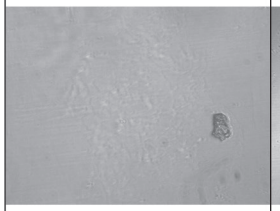
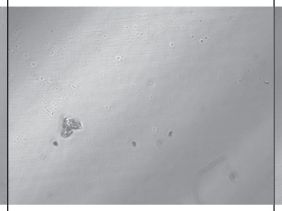
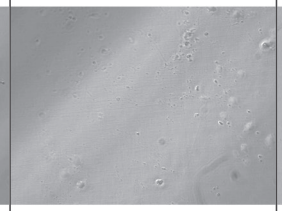
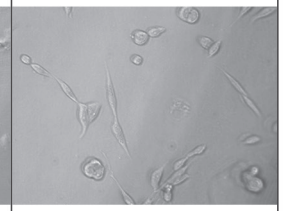
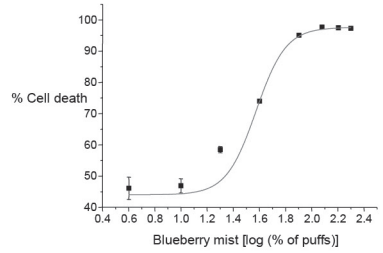
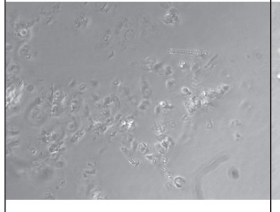
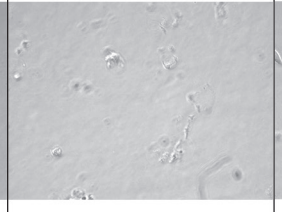
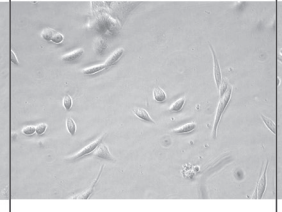
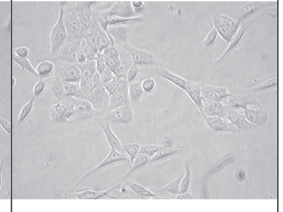
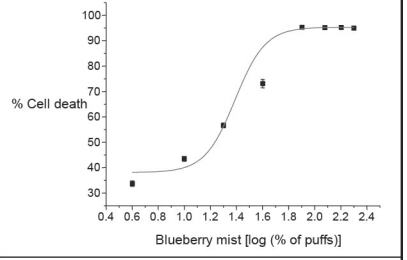
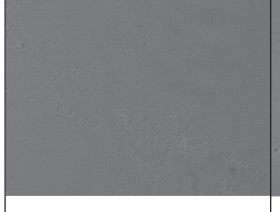
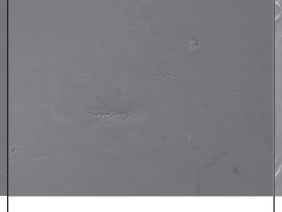
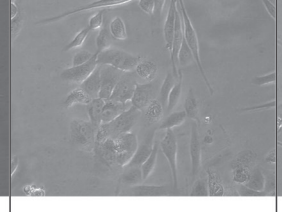
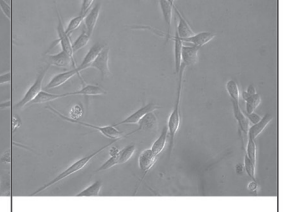
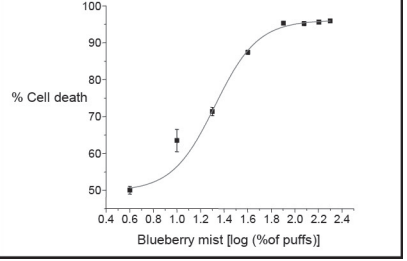
Vapouriz Strawberry Bliss

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

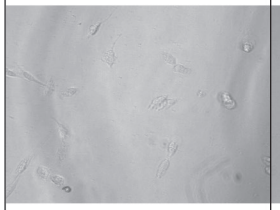
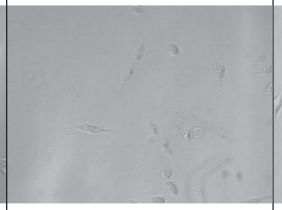
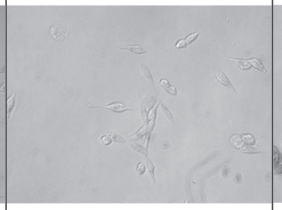
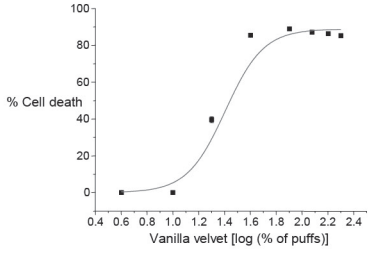
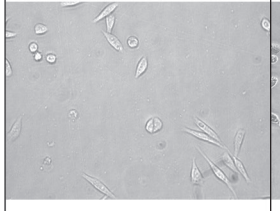
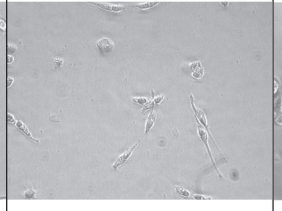
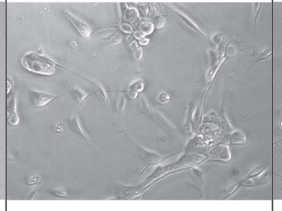
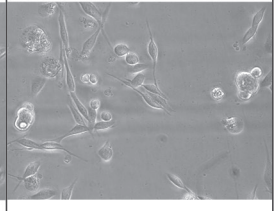
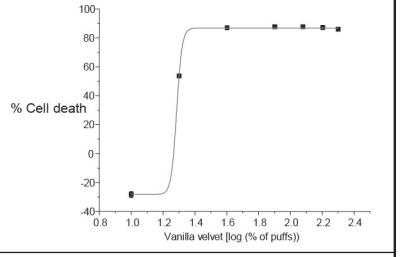
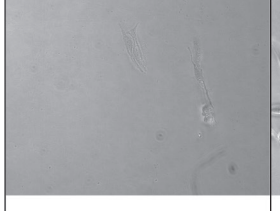
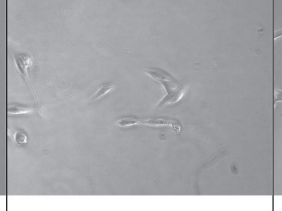
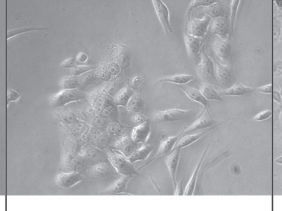
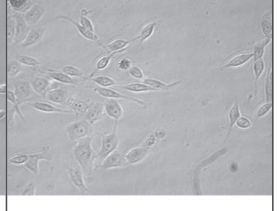
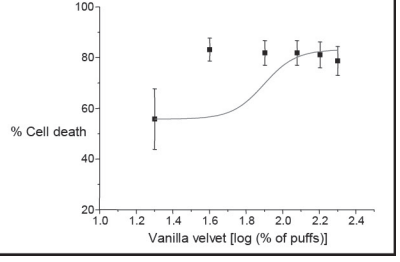
Nicolite Tobacco

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

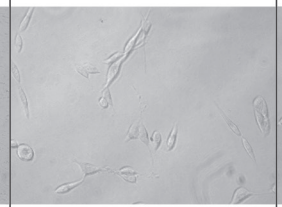
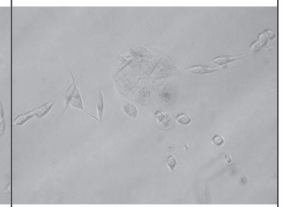
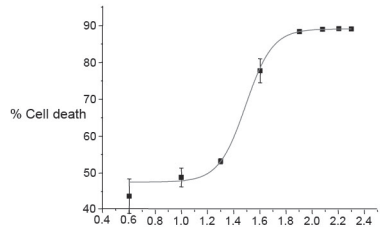
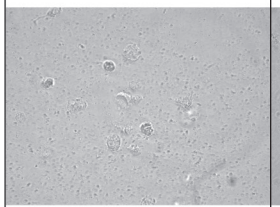
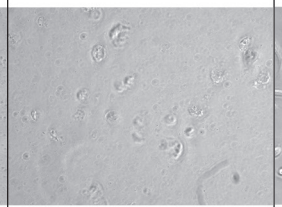
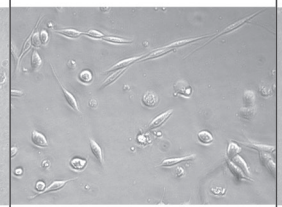
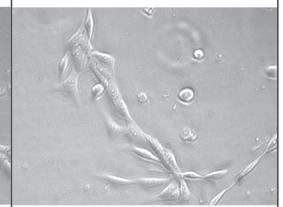
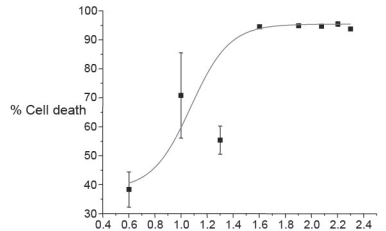
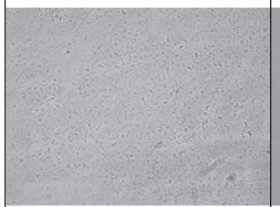
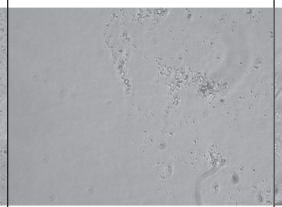
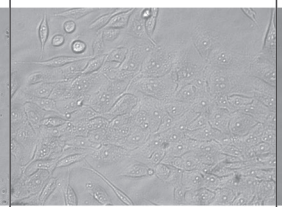
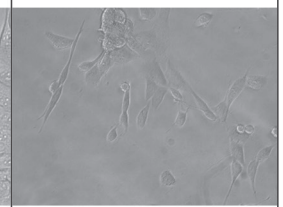
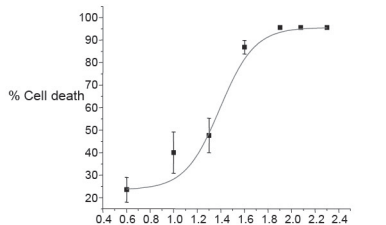
Dekang Blueberry Mist

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

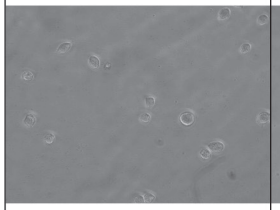
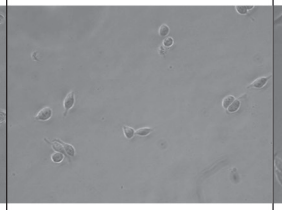
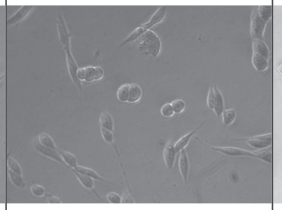
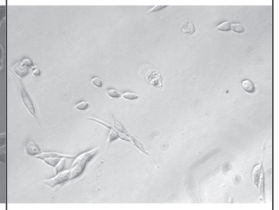
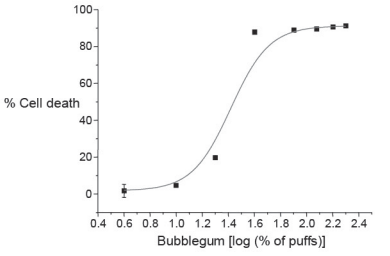
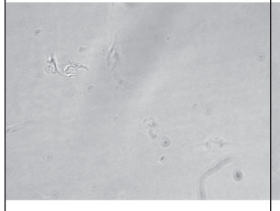
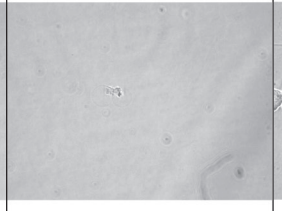
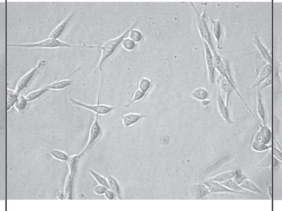
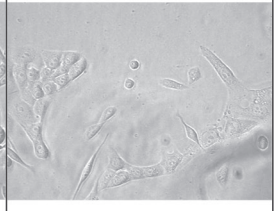
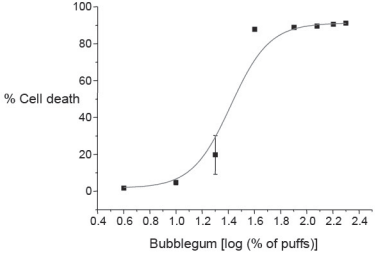
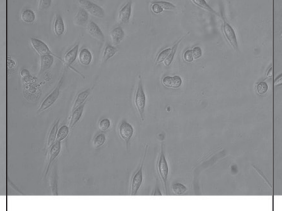
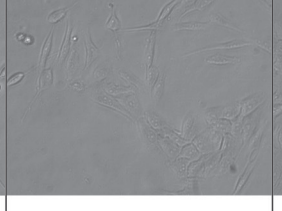
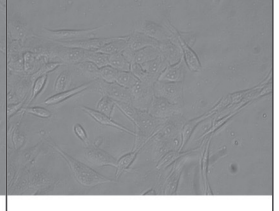
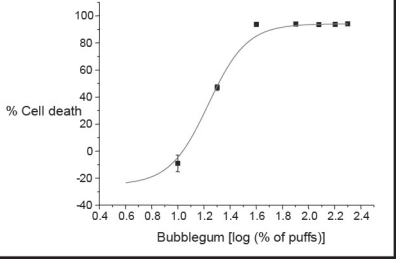
Vapouriz Vanilla Velvet

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

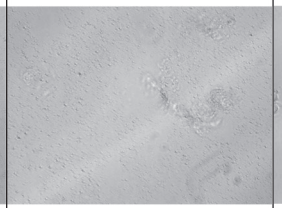
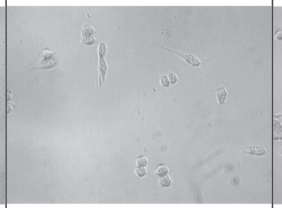
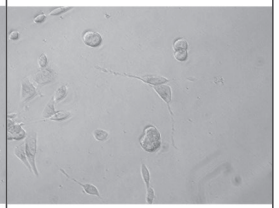
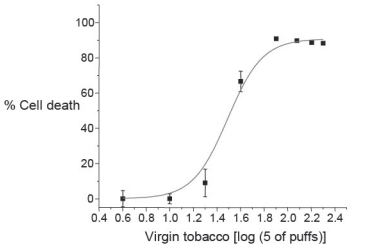
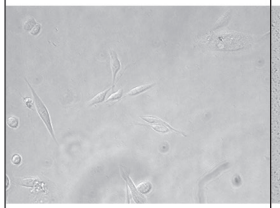
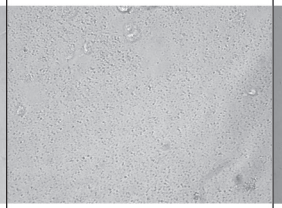
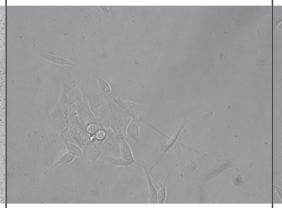
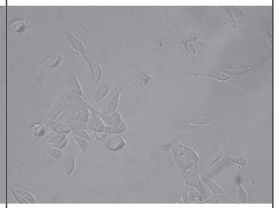
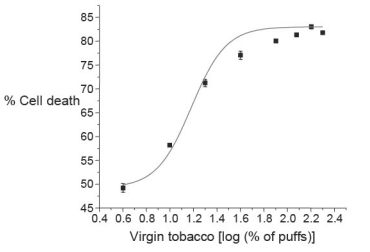
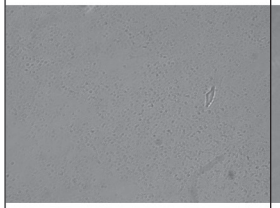
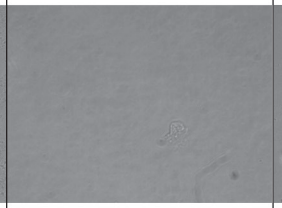
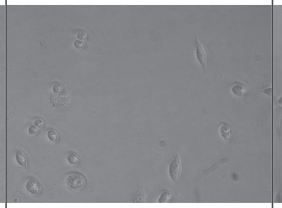
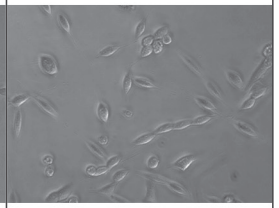
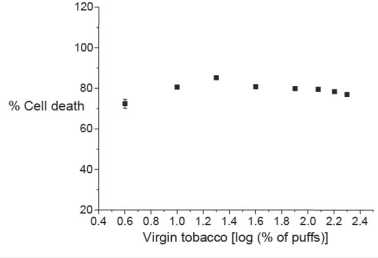
Nicolite Tobacco 16 mg mL^{-1}

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

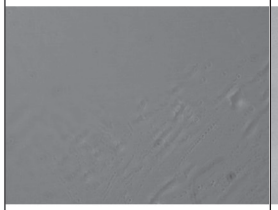
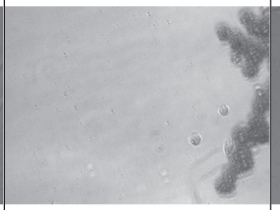
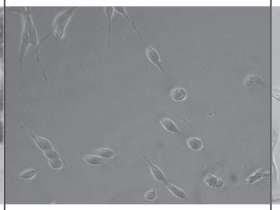
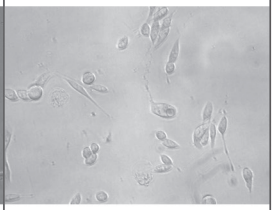
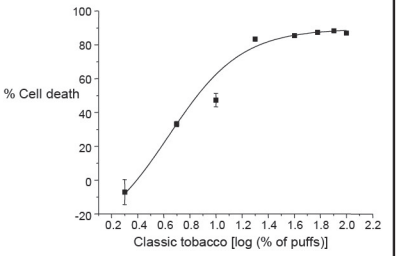
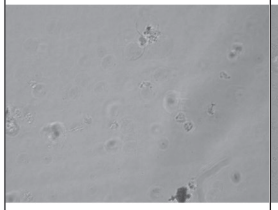
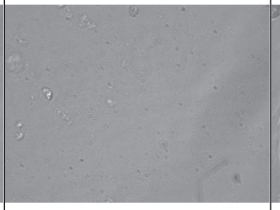
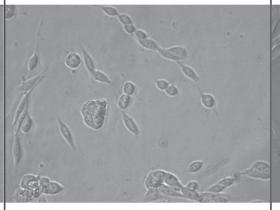
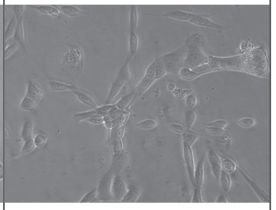
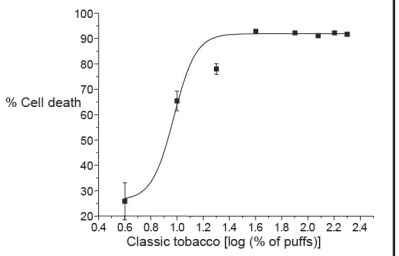
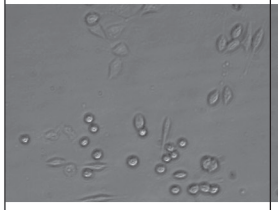
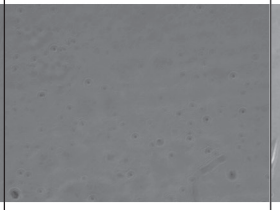
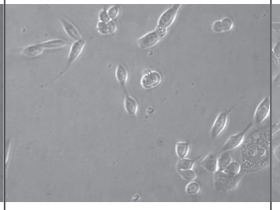
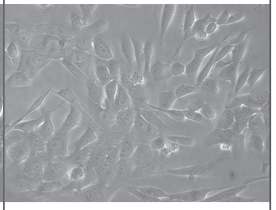
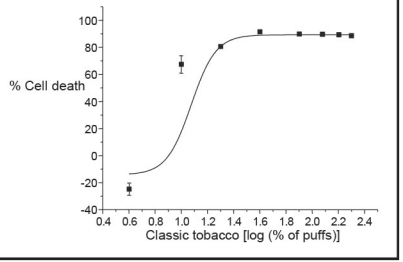
Vapouriz Bubblegum

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

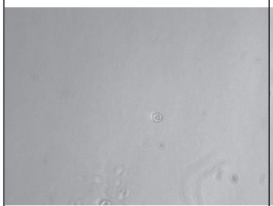
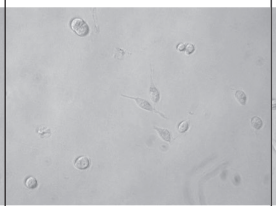
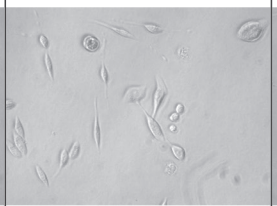
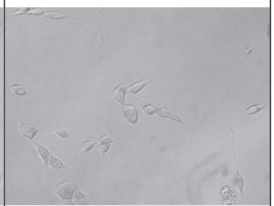
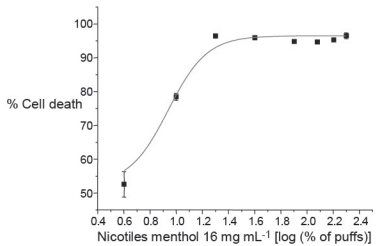
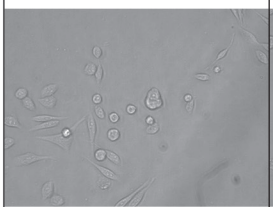
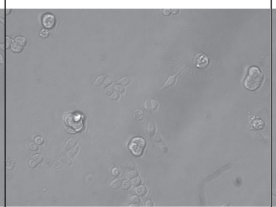
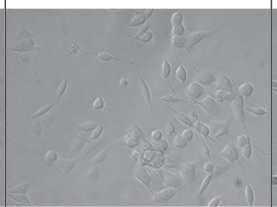
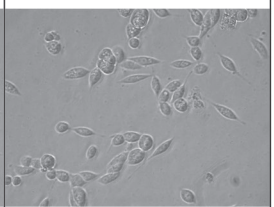
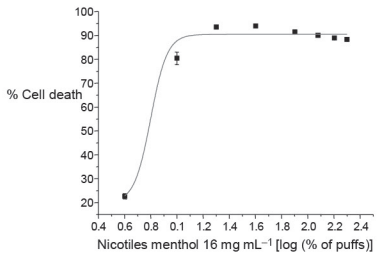
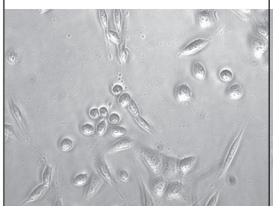
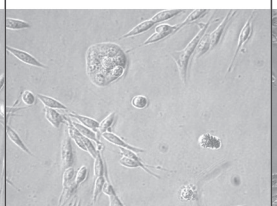
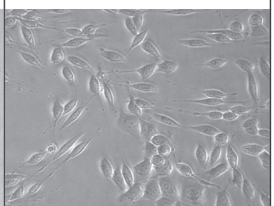
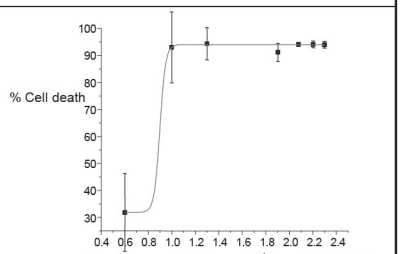
Vapouriz Virgin Tobacco

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

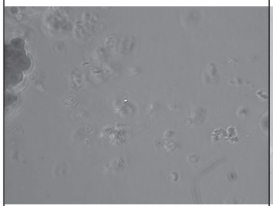
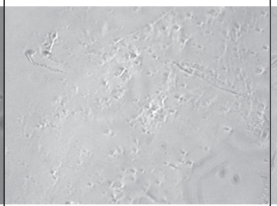
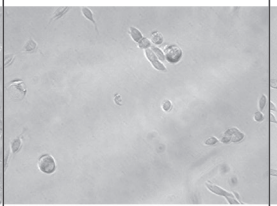
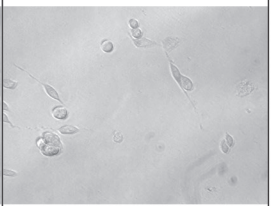
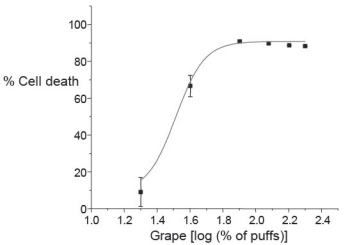
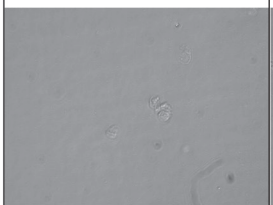
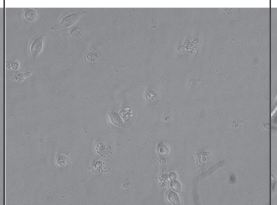
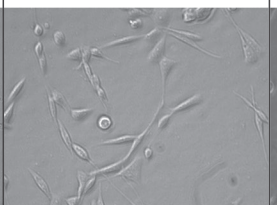
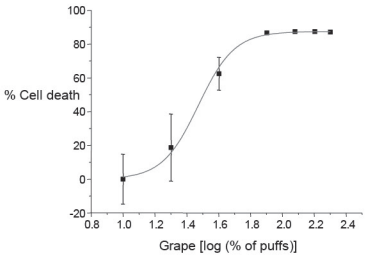
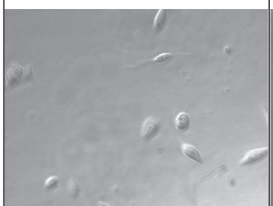
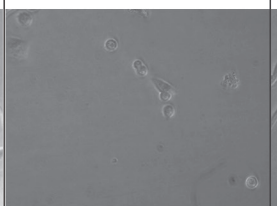
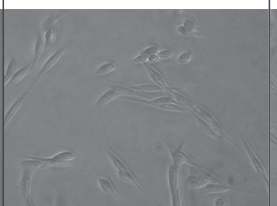
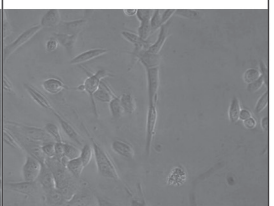
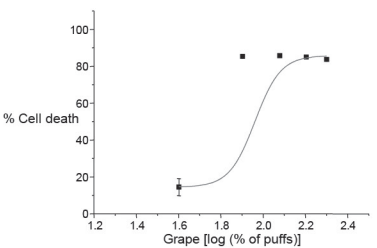
Vapouriz Classic Tobacco

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

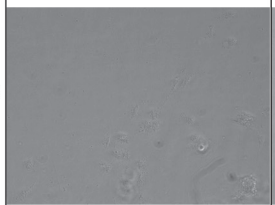
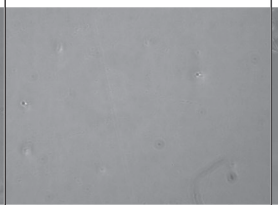
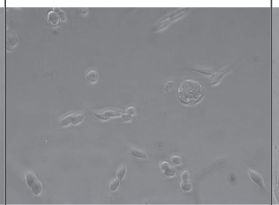
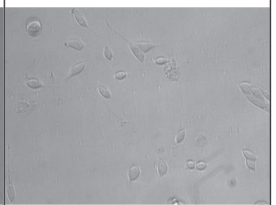
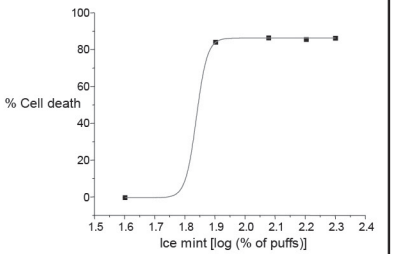
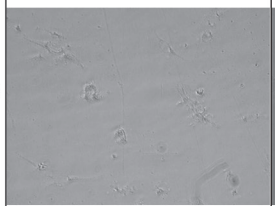
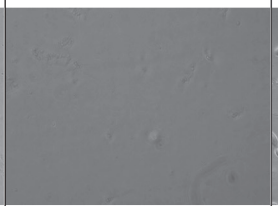
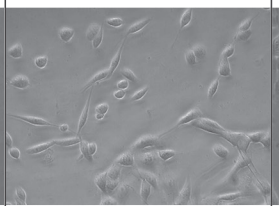
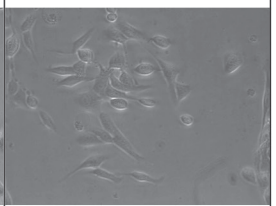
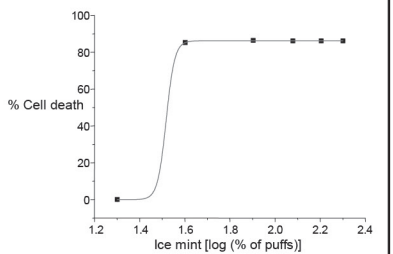
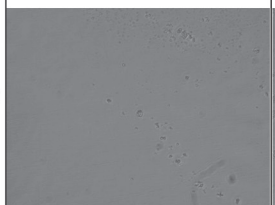
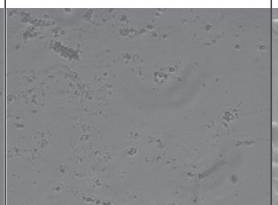
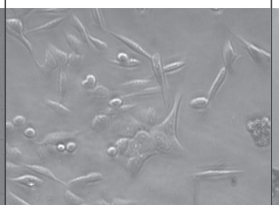
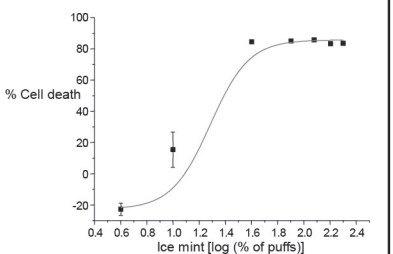
Nicolite Menthol

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

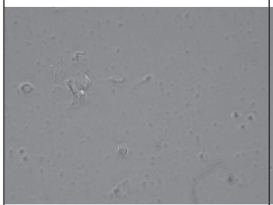
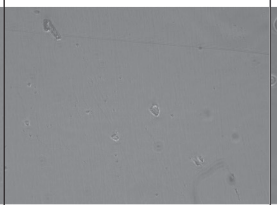
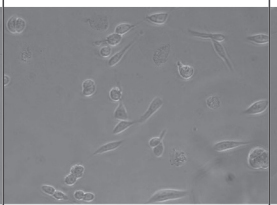
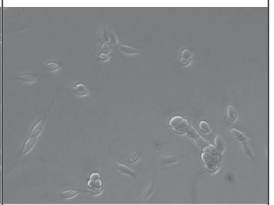
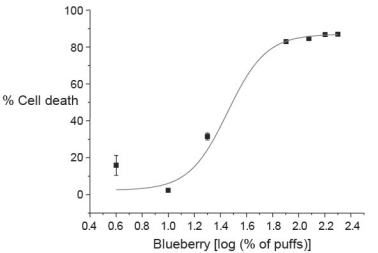
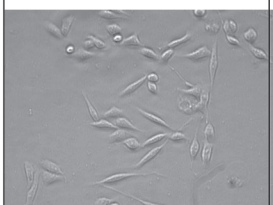
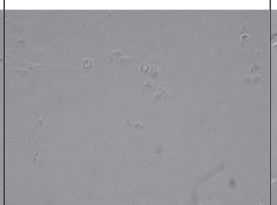
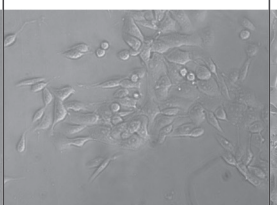
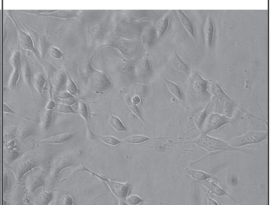
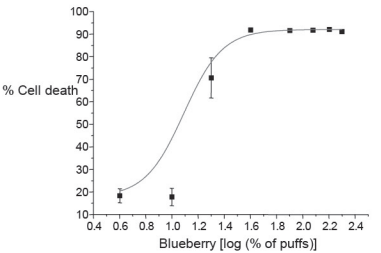
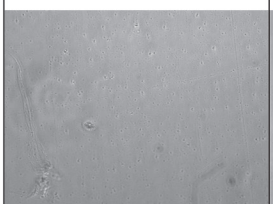
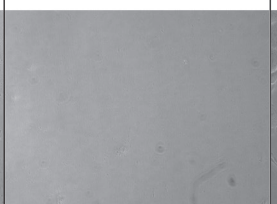
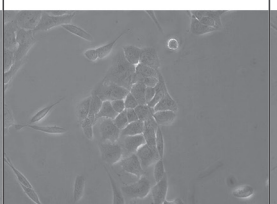
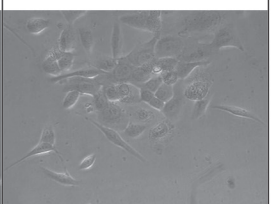
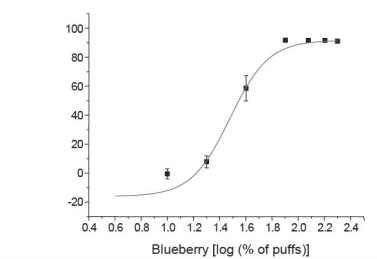
Vapouriz Grape

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

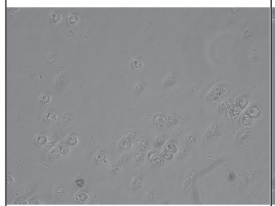
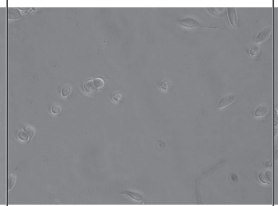
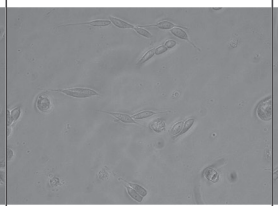
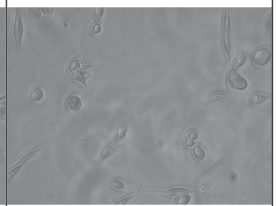
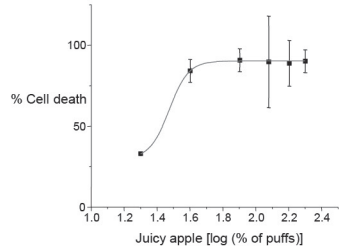
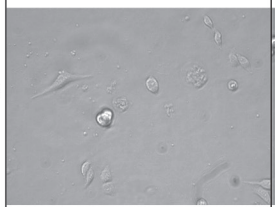
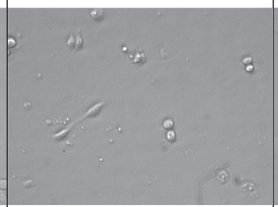
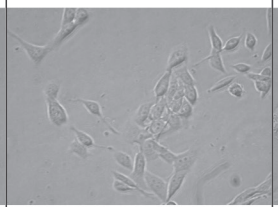
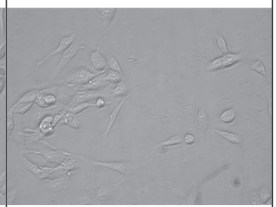
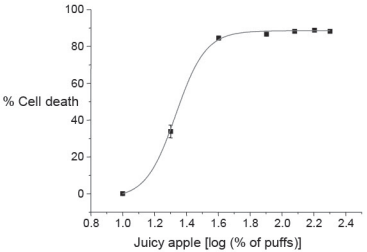
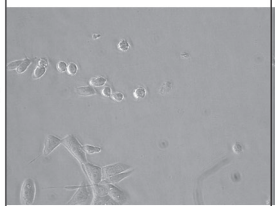
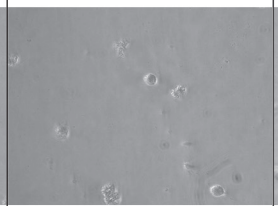
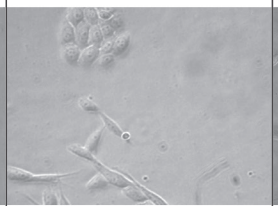
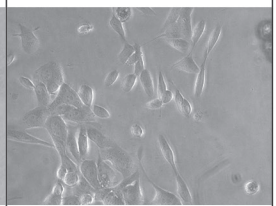
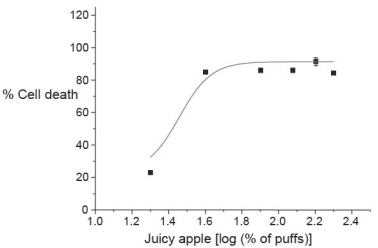
Vapouriz Ice Mint

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

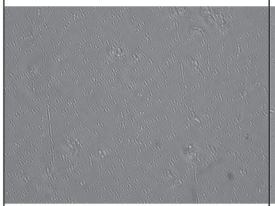
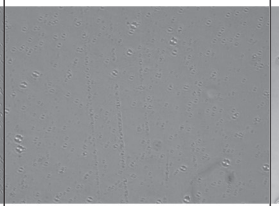
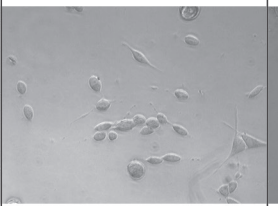
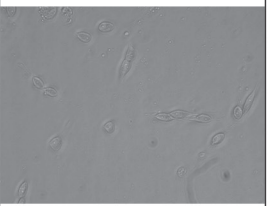
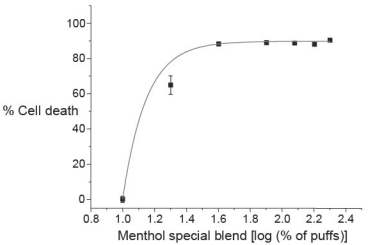
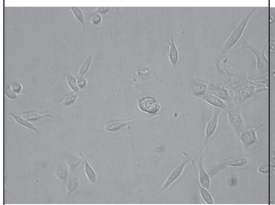
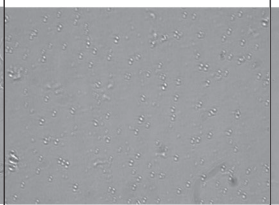
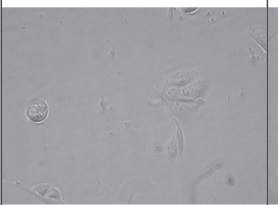
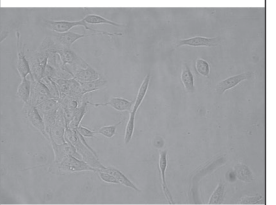
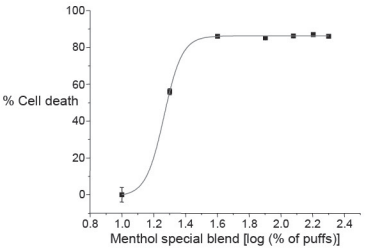
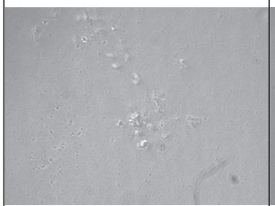
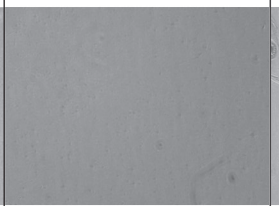
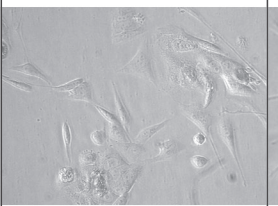
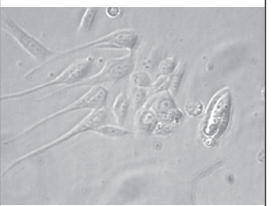
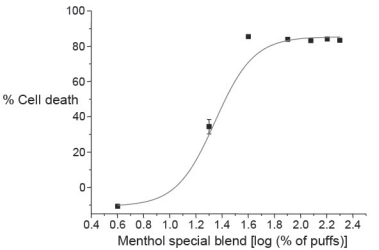
Vapouriz Blueberry

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

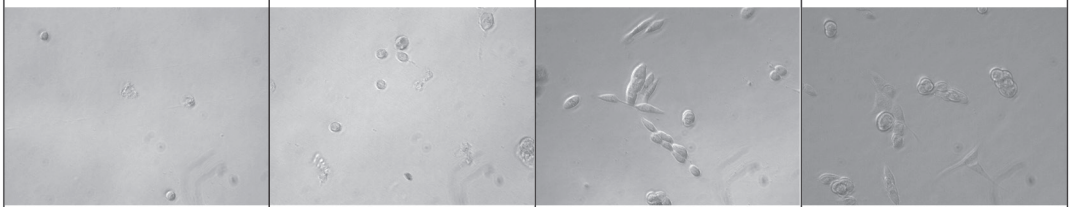
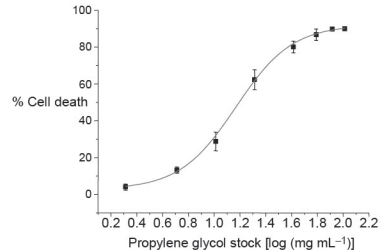
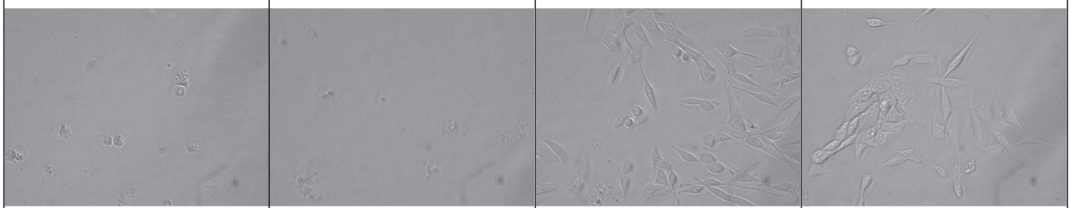
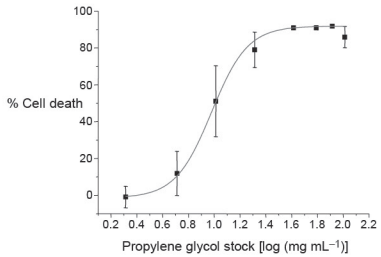
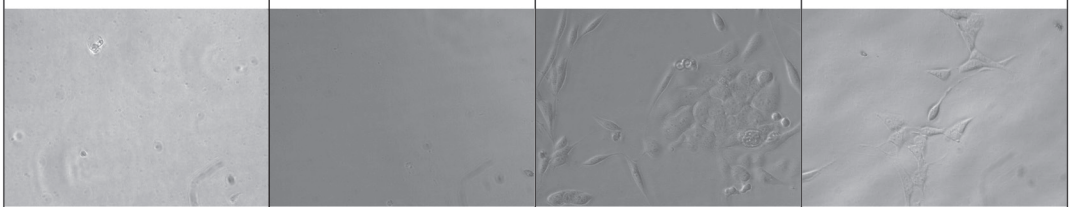
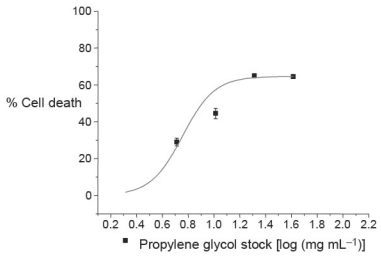
Vapouriz Juicy Apple

	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

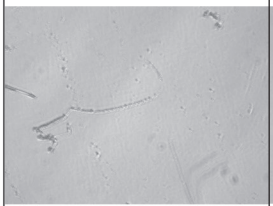
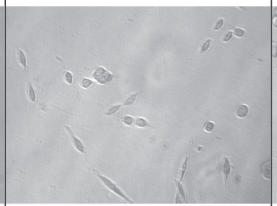
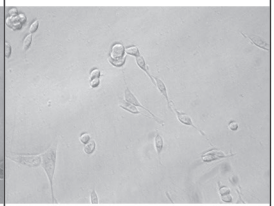
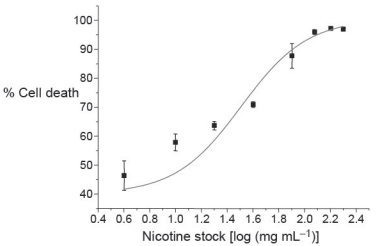
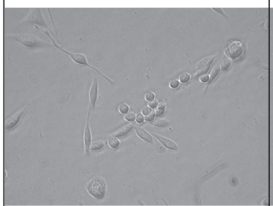
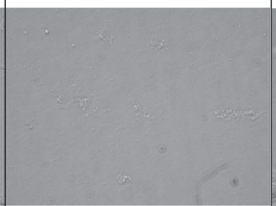
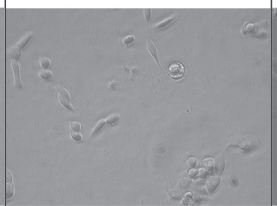
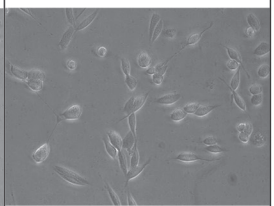
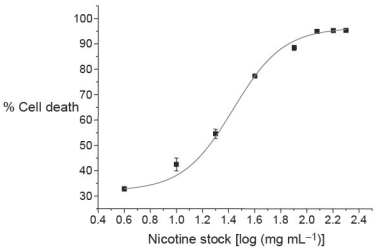
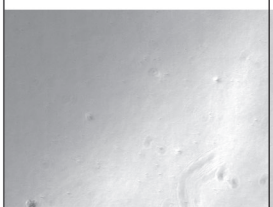
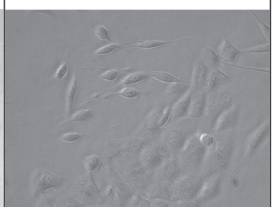
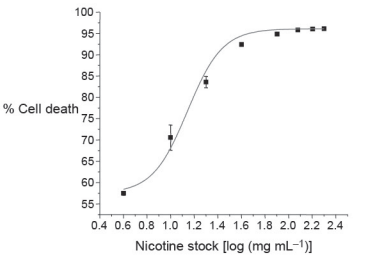
Vapouriz Menthol Special Blend

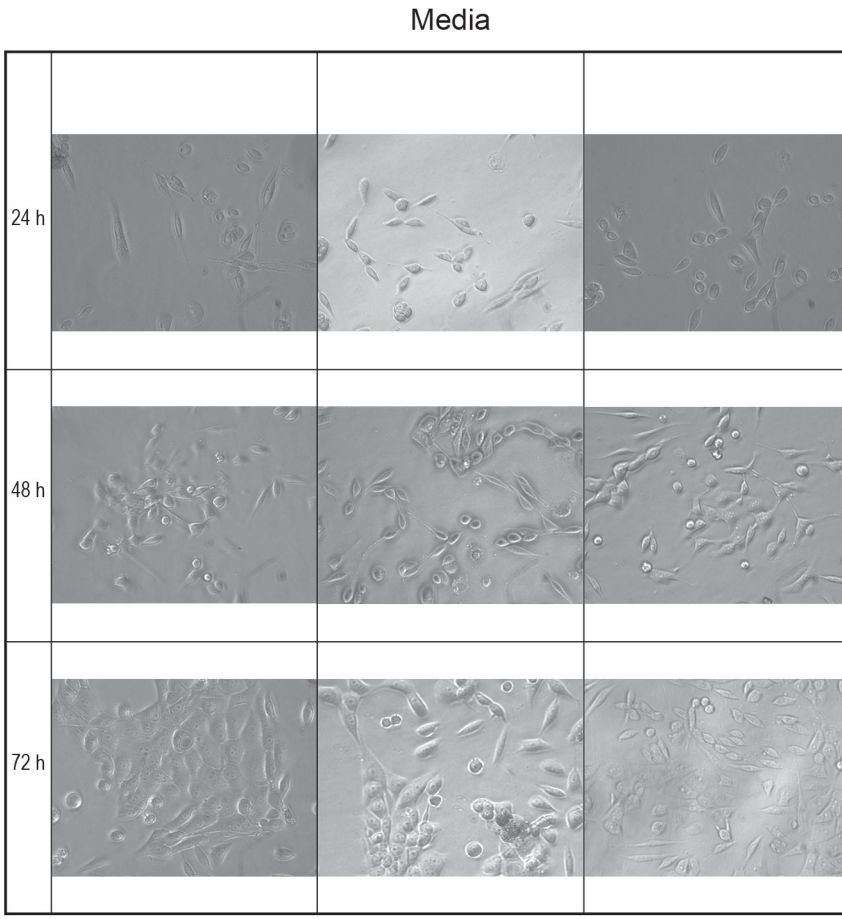
	200% of puffs (2 puffs)	120% of puffs (1.2 puffs)	10% of puffs (0.1 puffs)	4% of puffs (0.4 puffs)	IC_{50}
24 h					
48 h					
72 h					

Propylene glycol stock

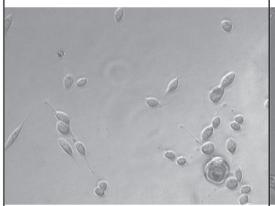
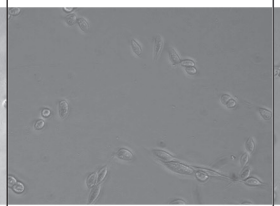
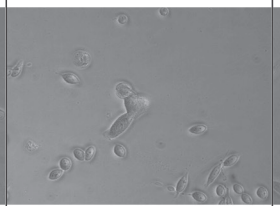
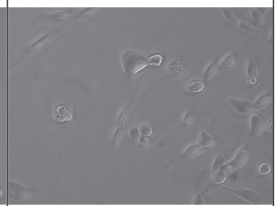
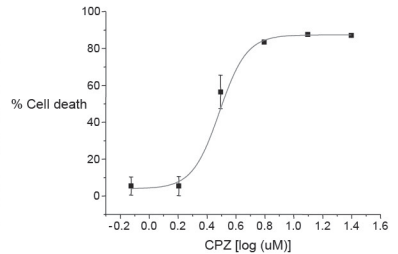
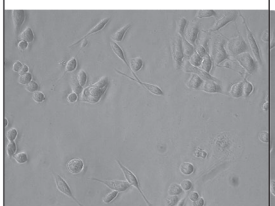
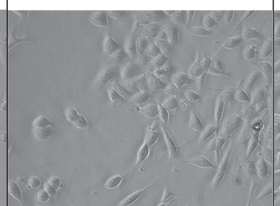
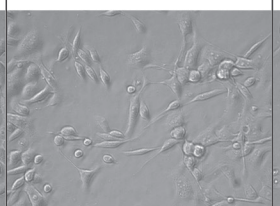
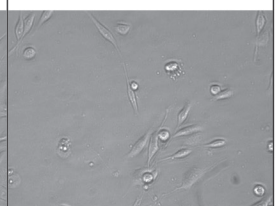
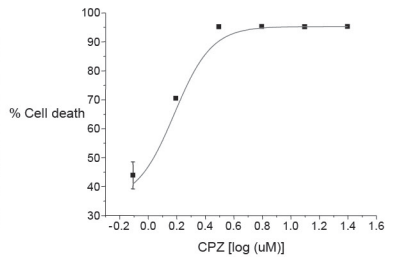
	0.1 g mL^{-1}	0.06 g mL^{-1}	0.005 g mL^{-1}	0.002 g mL^{-1}	IC_{50}
24 h					
48 h					
72 h					

Nicotine stock

	1.64 mg mL^{-1}	0.98 mg mL^{-1}	0.08 mg mL^{-1}	0.03 mg mL^{-1}	IC_{50}
24 h					
48 h					
72 h					


Media
24 h
48 h
72 h

Chlorpromazine

	50 µM	25 µM	12.5 µM	6.25 µM	IC_{50}
24 h					
48 h					
72 h